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ACTIVITY-PREVENTING AND EGG-SEA-WATER NEUTRALIZING SUBSTANCES FROM SPERMATOZOA OF ECHINOMETRA SUBANGULARIS

WALTER E. SOUTHWICK

(*From the Bermuda Biological Station for Research* [1])

Introduction

A spermatozoön, like all highly specialized biological units, has many utterly unique, but very significant characteristics. Of these, probably the most fundamental is the finiteness of its period of life. As has been clearly demonstrated by Lillie (1919), Gray (1928) and others, mature spermatozoa, except possibly those of certain arthropods, are unlike all other biological units in that they do not at any time ingest or otherwise assimilate substances from which energy may be derived. Once, therefore, that a spermatozoön has been liberated from the testis, its every power and activity must be accomplished on the basis of substances that were located in its structure at the time of its liberation. Studies to determine what different substances might be located in a spermatozoön, the location, nature, and approximate amounts of such substances, the changes in location and state which they might undergo, and the factors which might influence, affect, or effect such processes are, therefore, especially important for an understanding of spermatozoan physiology. A series of studies which were made at the Bermuda Biological Station for Research have provided some observations concerning such substances.

Materials and Procedures

The studies were made with the common reef urchin, *Echinometra subangularis*, individuals of which were obtained from the reefs on the eastern end of St. David's Island. The trip to the reefs was made by bicycle, and the urchins were brought to the laboratory in a pail with a small volume of water and covered with seaweed. The water

[1] Laboratory space for this investigation was provided by the Bermuda Biological Station for Research, Inc. during July, 1938.

was continually shaken, and so kept reasonably well aerated during the trip, a period of approximately twenty minutes.

Upon arrival at the laboratory, the urchins were immediately transferred to vessels in which a large volume of air was continually bubbled through a comparatively small volume of circulating sea-water. In such vessels, the urchins remained in good condition for approximately three days, and so trips were made regularly every two or three days in order to maintain a constant supply of freshly collected urchins.

The animals were opened by means of the usual circumferential cut, and the body fluids and intestines carefully removed. Every precaution was taken to avoid contamination, either of eggs with sperm, or of the sperm with egg secretions, and the gonads were removed from the test with glass needles. The ovaries were divided into small pieces in a small volume of sea-water with glass needles, and the mass strained through several thicknesses of unbleached cheesecloth. The supernatant fluid from the eggs was removed ten minutes later and fresh sea-water added. After two or three washings in this way, the supernatant fluid was allowed to stand for thirty minutes, at which time it would be capable of producing clear-cut and definite agglutinations with fresh sperm. The egg-sea-water was kept separate from the eggs during all subsequent tests. The testes were removed to a clean, dry Syracuse watch crystal, and, as fresh sperm was needed, the tubules were broken with a glass needle and the exuding dry sperm collected with a moderately fine pipette.[2] The watch crystal with the testes and the containers with the eggs or egg-secretions were kept covered at all times except when the materials were being withdrawn.

Movements of the Spermatozoa

When dry sperm is examined immediately under the microscope, the whole mass may be seen to be in a state of most intense vibratory activity. In such spermatozoa, however, due probably to the compactness of the mass, the active vibrations of the tails serve but to cause a rapid milling about of waving heads. Progressive movements are entirely absent, and the same group of spermatozoa sway back and forth, in constant, rapid vibration, but always in the same position, relative to each other and to the field of the microscope.

In such dry sperm, this motion lasts for from 50 to 120 seconds,

[2] Insemination tests made with such spermatozoa in a dilution proportion of one drop of dry sperm to 20 cc. sea-water, one drop of this suspension to one drop of eggs in 7 cc. sea-water gave, consistently, a fertilization percentage of from 92 to 100 per cent.

and then all movement ceases. The cessation in activity, once it has started, spreads rapidly, so that the entire transition from universal activity to complete quiescence is accomplished within about 15 seconds. Such spermatozoa do not show motion when they are redistributed mechanically by means of pressures on the cover glass.

When, however, a drop of sea-water is brought in contact with a drop of dry sperm, the edge of the drop of dry sperm "frays" slightly. After about one minute, the spermatozoa in this frayed edge begin to move slowly. Gradually, they come to move more rapidly, more spermatozoa become active, and they move slowly out into the drop of sea-water. In about two minutes, the whole mass, or as much of it as is reached by the diffusing sea-water, becomes intensely active. In the denser portions of this mass, as in the original drop of dry sperm, the motion is essentially a rapid milling about of waving heads with progressive movements entirely absent. In the less dense portions, however, progressive movements do occur.

When the dilution of one drop of dry sperm to one drop of sea-water is made upon a glass slide where such changes in the supporting medium as might be produced by evaporation are minimized by means of placing pure petroleum jelly around the edges of the cover glass, the activity continues, with gradually diminishing intensity, for about three or four hours, when all motion ceases. If another drop of sea-water then be added to the suspension under the cover glass, the spermatozoa again become active, and this activity continues with gradually decreasing intensity for about one and one-half hours, when all motion again ceases. Such spermatozoa can be reactivated again by the addition of another drop or two of sea-water, but the length of time during which the spermatozoa remain active with each successive reactivation progressively decreases until finally, after about eight such reactivations, no further activation is obtained. This phenomenon is shown more clearly in Table I which gives the actual observations of a typical experiment of this nature.

If the reactivation obtained from the addition of fresh sea-water to a suspension of inactivated spermatozoa were due to dilution effects, then one might expect a substance to occur in the suspensory fluid of an inactivated suspension of spermatozoa which would have the property of preventing dry sperm from becoming active. In order to test this possibility, a suspension of dry sperm was centrifuged for 30 minutes at 4,000 revolutions per minute. When this centrifuging had been completed, a clear, slightly opalescent, bluish fluid comprising about 11 per cent of the total volume of the original dry sperm had separated from the mass of spermatozoa. Utmost care had been

used to have the centrifuge tubes clean and *perfectly dry* before the spermatozoa were added, and great care was now used to have pipettes, glass slides, and cover glasses all perfectly dry. A drop of the clear supernatant fluid was removed from the centrifuge tube and carefully

TABLE I

Changes in the activity of a suspension of spermatozoa with time, and the effects of repeated additions of fresh sea-water upon the duration and changes in the activity of the spermatozoa in such suspensions.

Time	Observations on the Activity	Treatment of the Suspension	Number of the Sea-water Additions	Time Interval between Sea-water Additions
10:45		1 dr. dry sperm	0	
10:45	Intensely active			
10:47	All sperm utterly motionless	1 dr. sea-water	1	
10:48	Intensely active			
11:02	Active			
11:13	Some motion			
11:29	Some motion			
11:44	A little motion			
12:05	A little motion			
12:26	A little motion			
1:21	Still a little motion			
1:48	Just a little motion			
2:12	No motion	1 dr. sea-water	2	3 hours 27 min.
2:13	Intensely active			
2:39	Active			
2:49	Active			
3:27	Some activity			
3:53	Motionless	Sea-water added	3	1 hour 40 min.
3:57	Activation			
4:50	Motionless	Sea-water added	4	57 min.
4:52	Some activity			
5:12	Active			
5:51	Motionless	Sea-water added	5	61 min.
5:55	Some activity			
6:25	Active			
7:03	Motionless	Sea-water added	6	72 min.
7:05	Some activity			
7:47	Motionless	Sea-water added	7	44 min.
7:51	A little activity			
8:22	Motionless	Sea-water added	8	35 min.
8:30	No further activation			

brought in contact with a drop of dry sperm. The whole process of fusion of the two drops was carefully watched with the microscope, but there was no slightest sign of any activation whatsoever at any time. Even when the spermatozoa were thoroughly distributed throughout the supernatant sperm fluid by means of stirring with a

clean dry glass needle, there was no activation, and even examinations with a magnification of 950 × failed to show any activity whatsoever. Dilution of dry spermatozoa from the same testis tubules with ordinary sea-water gave perfectly typical activation.

Two preparations with the supernatant fluid were made permanent with the edges of the cover glass sealed with petroleum jelly, one at 12:35 P.M., and one at 1:55 P.M. The spermatozoa in the former were still motionless at 2:21 P.M. but when fresh sea-water was added at that time, the spermatozoa became intensely active by 2:25 P.M. In the latter, the spermatozoa remained motionless until 7:30 P.M., but when fresh sea-water was added at that time, became intensely active by 7:37 P.M. *The residual spermatozoa after centrifuging showed normal activation in all cases when ordinary sea-water was added.*

This experiment definitely indicated that the suspensory fluid of dry spermatozoa presents a condition which serves to prevent fresh dry sperm from becoming active. This condition occurs also, in the supernatant fluid, when 3″ of dry sperm is centrifuged for 30 minutes at 2,500 revolutions per minute through 1½″ of fresh sea-water. If the supernatant fluid from such centrifuging be removed and replaced with fresh sea-water, and the spermatozoa of the first centrifuging be centrifuged through this sea-water for 45 minutes at 2,500 revolutions per minute, the supernatant fluid from this centrifuging, too, will prevent the activation of dry sperm.

All gradations occur. All supernatant fluid obtained by any system of centrifuging dry sperm will prevent activation of fresh sperm. When more dry sperm than sea-water is present, one passage of the spermatozoa through the sea-water is sufficient to render the supernatant fluid capable of preventing activation of dry sperm. When, however, more sea-water than dry sperm is present, several washings, the number depending upon the condition of the spermatozoa and the relative proportions of sperm and sea-water, are necessary, and there are several experiments which demonstrate the relationship between the relative amount of dry sperm and sea-water present to the amount of centrifuging necessary to cause the supernatant fluid to prevent activity in dry sperm. Thus, in one typical experiment, dry sperm to sea-water in the proportions of ⅜″ : 3¼″, $1\frac{3}{16}$″ : 2¾″, 2⅜″ : 2½″, and 1⅞″ : 1½″ were each washed five times, being centrifuged approximately 10 minutes at 3,550 revolutions per minute for each washing. The supernatant fluid from the ⅜″ : 3¼″ proportion did not stop dry sperm, whereas the supernatant fluid from each of the other proportions stopped dry sperm completely. In another typical experiment, the proportion of dry sperm to sea-water was made the same

in all four tubes, namely ½″ : 3″, and a tube removed after 5, 10, 15, and 20 washings of approximately four minutes at 3,250 revolutions per minute for each washing. The supernatant fluid after five washings did not stop dry sperm, the fluid obtained after 10 washings allowed "just a little activity" in dry sperm, while the supernatant fluid obtained after 15 and 20 washings completely prevented activity in dry sperm.

In order to make certain that the occurrence of the condition in the supernatant fluid was developed by the centrifuging, an exactly similar proportion of dry sperm and sea-water was made and thoroughly mixed at the same time that the other tubes were prepared. This suspension of spermatozoa was kept in a tube until the centrifuging of the other tubes had been completed. The sperm in this control tube were then separated from the suspensory fluid by means of one centrifuging at 3,250 revolutions per minute for 8 minutes, and the resulting supernatant fluid tested. It was found to be utterly incapable of preventing or reducing activity in dry sperm to any extent that could be optically determined.

These experiments indicate that some substance or condition develops or appears in suspensions of spermatozoa which has the property of preventing activity in the spermatozoa of that or of a fresh suspension of dry sperm. This substance or condition is rendered ineffective, or is reduced to sub-threshold concentration or intensity by the addition of fresh sea-water, but its concentration or intensity may be definitely and markedly increased by washing the spermatozoa through the suspensory fluid by means of the centrifuge.

Other Properties of Supernatant Sperm Fluid

When a drop of supernatant fluid which will definitely prevent activity in dry sperm is thoroughly mixed, by means of a clean, dry glass needle, with one drop of an egg-sea-water which will produce definite and clear-cut agglutination clumps with dry sperm and this mixture, in the form of a drop on a clean, dry glass slide, is brought into contact with a drop of dry sperm from the same tubules, and the whole process watched carefully with the microscope, the spermatozoa will be seen to become active in a manner exactly similar to that which occurs when a dilution is made with ordinary sea-water, but no slightest sign of any form or degree of agglutination whatsoever occurs. This test was repeated several times with many different preparations, and with many different lots of gametes, but always with the same result. In all cases when the supernatant sperm fluid would prevent activity in dry sperm, it would neutralize or destroy in some way the ag-

glutinating power of egg-sea-water, but reciprocally, its power to prevent activity in dry spermatozoa was similarly destroyed, and the mixture of supernatant fluid plus egg-sea-water served to activate spermatozoa in a manner exactly similar to that of ordinary sea-water.

This phenomenon was even more vividly shown when a small drop of the supernatant sperm fluid was added, with a fine capillary pipette, to the middle of the field where the agglutinated clumps, produced by the addition of egg-sea-water to dry sperm, could everywhere be seen, and the whole process watched continuously under the microscope. The response was almost instantaneous. The agglutination clumps everywhere within the area of the added drop *immediately* dispersed, while outside the boundary of the drop and on all sides, clumps persisted in a perfectly typical and unaffected arrangement. Within the drop, the spermatozoa instantly became inactive, while along the boundary of the drop, some activity and motion could be seen.

The phenomenon, too, was readily demonstrated by making preparations on one slide which could be observed successively and sequentially, and directly compared, and which would consist of dry sperm mixed with (1) ordinary sea-water, (2) egg-sea-water, (3) supernatant sperm fluid, and (4) one drop egg-sea-water plus one drop supernatant sperm fluid. (1) and (4) showed typical activation, but no agglutination, (2) showed distinct and definite agglutination, while the spermatozoa in (3) were utterly inactive.

There seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water. All supernatant fluid that would prevent activity in dry sperm would also neutralize the agglutinating power of egg-sea-water. When the supernatant fluid allowed "just a little activity," the mixture of supernatant sperm fluid and egg-sea-water allowed "slight and evanescent tendencies" towards the formation of agglutination clumps, while finally, the supernatant fluid obtained from a sperm suspension that had been diluted for 2¾ hours, but that had not been washed by centrifuging, would not prevent activity of dry sperm, and would not prevent egg-sea-water from causing agglutination. These observations seem to indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant sperm fluid are due, either to the same substance or condition, or to two or more substances which, however, arise or occur together in the supernatant fluid under all the treatments where the effects have been observed.

Discussion

Attempts to extract such substances from the spermatozoön as would have a relationship to the fertilization reaction have been

made by Sampson (1926), Hibbard (1928), Wintrebert (1929, 1930*a*, 1933), Einsele (1930), and Parat (1933*a*). Sampson was unable to obtain any substances in sperm filtrates and dialysates which would activate "fertilizin" in the egg-sea-water so as to make it an efficient parthenogenetic agent, or would combine with the agglutinating substance in egg-sea-water so as to destroy its power to agglutinate fresh sperm suspensions, though she did obtain substances which would initiate development of mature ova of the same species. Hibbard and Wintrebert found that solutions of macerated spermatozoa would digest egg membranes, while Einsele and Parat found that filtered ether dialyzates and extracts of entire testes would, when injected into the egg with a micropipette, give an activation of the eggs in 60 per cent of the cases. Parat found that the development was much more regular, and that many more of the gastrulae would form larvae when the eggs were activated parthenogenetically by means of the introduction of the acrosome of spermatozoa removed and injected by means of microdissection needles and pipettes. Both Parat and Einsele have obtained evidence that the substance concerned in such parthenogenetic activations is a proteolytic enzyme.[3] Histo-anatomical studies of spermatozoa have been made by Bowen (1924), Popa (1927), Wintrebert (1930*b*), Parat (1928, 1933*b*) and others, and some of these studies indicate the presence of substances of secretory origin in the acrosomal region of spermatozoa.

Since egg-sea-water, reciprocally, will neutralize or destroy the activity-preventing or inhibiting substance of a sperm suspension, the activating property of egg-sea-water, as described by Lillie (1913), hereby receives an explanation. The power of the substances in the supernatant sperm fluid to neutralize the agglutinating power of egg-sea-water also conforms very readily with the fertilizin theory of Lillie (1919), and with the observations of Lillie (1919) and others with *Arbacia punctulata* that it was not possible at any time to regain agglutinating substances that had once been used in order to cause an agglutination of spermatozoa.

Summary

1. Dry sperm of *Echinometra subangularis* is intensely active immediately after its removal from the testes tubules, but this motion

[3] Recently (1939), Frank has described a sperm extract of *Arbacia punctulata* spermatozoa, obtained by heating the sperm suspension, which has the property of neutralizing the fertilizin of egg-sea-water, and which has an agglutinating effect on the cilia of *Arbacia* plutei. It is possible that the inactivating effect of the supernatant fluid of *Echinometra* spermatozoa is due to a similar action upon the tails of the sperm.

lasts for but from 50 to 120 seconds, when all activity ceases. When, however, a drop of sea-water is brought in contact with such a drop of dry sperm, the spermatozoa again become active, and this activity continues for from three to four hours. The addition of another drop of sea-water will cause the spermatozoa again to become active, and this activity continues with gradually decreasing intensity for about one and one-half hours. Such spermatozoa can again be reactivated, but the length of time during which they remain active progressively decreases, until finally, no further activation is obtained.

2. This inactivation of sperm suspensions with time is caused, probably, by the accumulation of a substance with time which has, as its characteristic identificatory property, the prevention of spermatozoa from activity. It occurs in the supernatant fluid obtained from centrifuging dry sperm, and in the supernatant fluid obtained by washing dry sperm through ordinary sea-water several times with the centrifuge, but the suspensory fluid of diluted sperm suspensions through which the spermatozoa have not been washed does not contain the substance in detectable amounts.

3. Supernatant fluid which contains this substance also, in all cases, has the property of neutralizing the agglutinating power of egg-sea-water, and there seems to be a parallelism between ability to prevent activity of dry sperm and ability to neutralize egg-sea-water.

4. These observations indicate that the activity-preventing and the egg-sea-water neutralizing properties of supernatant fluid are due, either to the same substance, or to two substances which, however, occur together under all the treatments where the effects have been observed.

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THE "AGGLUTINATION" PHENOMENON WITH SPERMATOZOA OF CHITON TUBERCULATUS

WALTER E. SOUTHWICK

(From the Bermuda Biological Station for Research [1]*)*

Introduction

It has been shown by Crozier (1922) that when sperm, diffusing from a male individual of *Chiton tuberculatus* during the month of May (fully a month before ripe eggs are seen), was taken up between the ctenidia of a female, it issued from the posterior ends of the ctenidial channels principally in the form of "numerous agglutinated masses of active sperms" which persisted in sea-water for at least one half-hour. He found similar "agglutination" when sperm had passed through the ctenidial channels of males, and when it had been added (1) to ovarian extracts from mature eggs in sea-water, or (2) to sea-water into which ripe eggs had been shaken from an ovary and allowed to stand for half an hour. He considered these conditions to indicate that "mere evidence of sperm agglutination (cluster formation) may well have no bearing on the fertilization reaction." This conclusion is in distinct conflict with that formulated by Lillie (1919) on the basis of his observations on *Arbacia punctulata* and *Nereis limbata*.

The observations of Crozier (1922) have been extended during a series of observations made during the summers of 1933 and 1938 at The Bermuda Biological Station for Research.

Observations

Dry spermatozoa [2] from a mature male *Chiton tuberculatus* are homogeneously motile. When such dry sperm is introduced into the mantle cavity either of a male, or of a female which does not shed eggs, in a way such that it is caught up in the ctenidial current and carried

[1] This investigation was supported in part by a grant from the Porter fund. Laboratory space was provided in part by Harvard University and in part by the Bermuda Biological Station for Research.

[2] When a drop of such sperm is brought into contact with a drop of mature eggs on a glass slide, the egg contents, within about one minute, shrink visibly so that a clear area is produced around the egg and between the egg and the chorion. When such eggs are transferred to Syracuse watch crystals containing approximately 10 cc. sea-water, about 98 per cent of them will have cleaved, within approximately one and one-half hours, to form the two-cell stage.

through the gills to be discharged at the posterior end, this discharged sperm, if allowed to collect in the dish, or to rest undisturbed on a glass slide, can be seen to form macroscopically visible white masses in a short time, as Crozier observed. If, on the other hand, the discharged sperm is collected with a clean pipette, transferred to a glass slide, and examined immediately, a very interesting series of changes can be observed.

During the first few seconds, the spermatozoa swim about actively, freely, homogeneously. During the next few seconds, they come together and form small clumps of spermatozoa, *but the point of attachment is the tail*, while the heads remain perfectly free. Sometimes the group may consist of but ten or twelve spermatozoa, in which case the clumps resemble bouquets of flowers, tufts of grass, or even more appropriately, a handful of balloons waving in the breeze. Soon these clumps fuse into larger masses, either in such a way as to form complete spherical masses, as shown in Fig. 1, or much more frequently, to form strands of spermatozoa. These strands, at first, are slender and comparatively short, and are often but slightly branched, but soon they elongate, thicken and branch, as shown in Fig. 2. Soon these strands fuse with others, in a way such that within about three minutes extensive networks appear, as may be seen from Fig. 3. These networks soon become so large that they are macroscopically visible.

Careful observation of the mode of formation of these strands reveals that the process is quite comparable to a braiding of the tails of the spermatozoa. Always at the end of the strand, a tuft of spermatozoa with entirely free heads may be seen. These heads are continually waving back and forth, in and out, moved by the whipping motion of the spermatozoön tail. In a short time, the latitude of the motion becomes restricted, and the restriction progressively increases until finally only the head is free. It can then be seen, still actively waving, from the side of the strand. Often, however, the head is included in the braid.

As the sperm become more and more bound in the strand, other spermatozoa get caught and soon these are inescapably bound while others are caught. This continues until all of the free-swimming spermatozoa are bound, when the terminal tufts then persist for long periods of time, probably permanently. These tufts have been examined with especial care, and in every case, the sperm head has been found to be absolutely distinct and completely separate from its neighbors.

When, however, a drop of dry sperm on a glass slide is brought in contact with a drop of sea-water in a way such that the spermatozoa

are carried into the sea-water by the resultant currents, it frequently happens that the sperm suspension is simply swept into the sea-water drop. Unlike the condition with most forms, the sperm mass tends to remain intact, and though it generally exhibits a slight increase in homogeneous motility, it shows no slightest sign of the clumping phenomenon. Occasionally, however, under such conditions, tufts of spermatozoa may form along the contacting edge, as may be seen in Fig. 4.

On the other hand, when the two drops are fused in a way such that the spermatozoa are distributed widely and rapidly into the sea-water, *all* stages that were observed with the sperm that had passed through the ctenidial channels could be seen repeated under such conditions. The phenomenon was first noted when the sperm drop was brought into contact, in the usual way, with a drop of sea-water to which a little ether had been added. The markedly reduced surface tension produced violent currents which served rapidly to carry the sperm to all parts of the drop. The ether evaporated rapidly, and by so doing probably produced still more currents, but did not appear to affect the motility of the sperm in any way. Later it was possible to repeat exactly the same series of changes with non-etherized, normal sea-water by the proper regulation of the relative sizes of the drops. When the two drops are fused with the aid of a clean glass needle in a way such that the sperm suspension is spread widely throughout the drop of sea-water, the typical strands, as shown in Fig. 2, form immediately and everywhere, and these soon anastomose to form the extensive net-works shown in Fig. 3. In these nets, the sperm heads frequently project into the interstices of the net and there continue to wave actively for a long period of time. Sometimes the network formation may be so extensive that distinct membrane-like structures are produced, which readily curl up and may readily be caused to wave in a manner typical of any such membrane, if the slide be moved or shaken gently under the microscope.

When a drop of dry sperm is introduced suddenly into 8 or 10 cc. of sea-water in a small beaker, the sperm mass may be seen to drop to the bottom of the beaker in the form of a much-folded membrane, resembling in every respect a piece of silk allowed to fall lightly on a table. Microscopic examination of this mass shows that it is composed of a still intact mass of homogeneously distributed spermatozoa. Though this membrane of spermatozoa is very delicate, it is possible to lift it, *as a membrane*, with a fine glass needle, and to fold it back upon itself, or to roll it into a markedly more compact mass. Tufts, in due time, appear along the edges of the mass, and circular clumps may

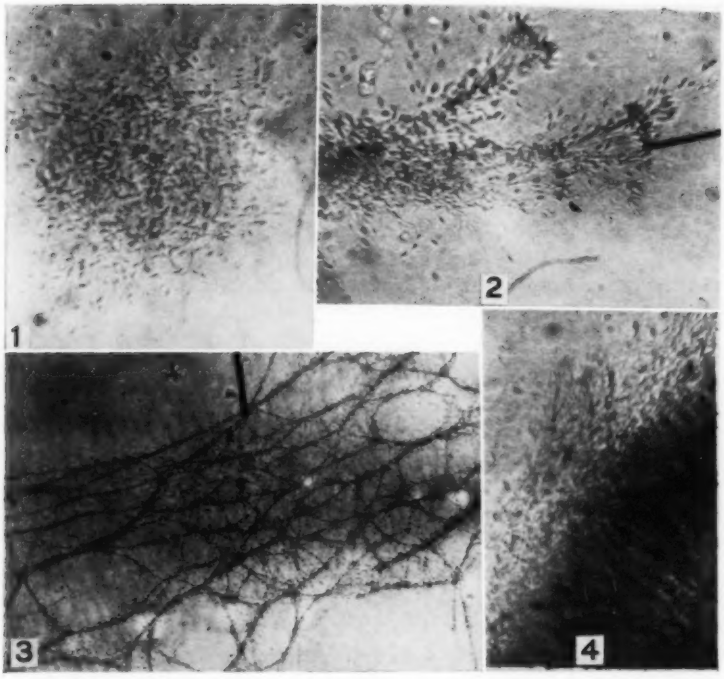

PLATE I

FIG. 1. The structure of the spherical masses that are formed by the spermatozoa of *Chiton tuberculatus* after dilution with sea-water, or after passage through the ctenidial chambers of a male or immature female. Sometimes the masses formed are more compact, but the structure, with the tails bound and the heads free, is the same in all cases. × 440.

FIG. 2. The elongating, thickened, branching strands that form when the spermatozoa of *Chiton tuberculatus* is passed through the ctenidial chambers of a male or immature female, or is diluted with sea-water. × 440.

FIG. 3. The nature of the network that forms by the fusion of the strands produced by the spermatozoa of *Chiton tuberculatus* when diluted with sea-water, or passed through the ctenidial chambers of a male or immature female. × 100.

FIG. 4. Photomicrograph to show the retention of mass integrity, with occasional formation of tufts along the contacting edge, when a drop of dry spermatozoa of *Chiton tuberculatus* is gently brought into contact with a drop of sea-water. × 440.

appear separated from, but near the margins of, the membrane. Rarely, strands may also appear. When, however, the original sperm mass becomes spread very thin, widespread formation of the network usually results.

If, however, some of the sperm be separated from the main mass, either by means of a glass needle, or by mechanical agitation, typical strands and networks are immediately formed. Any mechanical disturbance of the drop of dry sperm in order to obtain a uniform, homogeneous, diluted sperm suspension results only in a complete transformation of the sperm mass into strands, and a microscopic examination of sperm suspensions of one drop of dry sperm to 5, 10, 15, 25, 50, 100, and 150 cc. sea-water have all shown the presence of such strands, and the complete absence, in any case, of completely free swimming spermatozoa. These strands slowly settle to the bottom of the container, the rate depending upon their size, where they form into networks, and, if the concentration be sufficient, into more or less homogeneous membranes. These membranes can again be broken up into strands, and the process can be repeated several times. The strands in sea-water persist indefinitely, or as long as the spermatozoön tail is intact. In the more dilute suspensions, however, the strands remain quite uniformly distributed in the suspension for some time.

On the other hand, when extracts from the ovaries of ripe or spent females, obtained by cutting the ovary into small pieces and washing it thoroughly through cheese-cloth with about 25 cc. of sea-water, are used instead of sea-water, *no clumping in any form occurs*. Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop is reached. At no time do tufts, strands, or networks appear, and, furthermore, if spermatozoa be drawn from the mass by means of a glass needle, they simply disperse and soon merge imperceptibly with the other spermatozoa as the main mass advances upon and closes in about them.

Dilution of the ovarian extracts appears to lessen their effectiveness. A dilution of one drop of extract to ten drops of sea-water is often quite as effective as the full strength extract. A 1 : 25 dilution, however, prevents the formation of tufts and other similar structures, but does not remove the restriction to a free movement of the sperm through the drop of ovarian extract; while a 1 : 50 dilution allows the formation of tufts, clumps, strands, and networks just as though it were pure sea-water. These figures, however, are of relative value only, since there is a wide variation between the extracts from the different ovaries, as seen in the fact that one of those tested gave a perfectly

typical clumping reaction with a 1 : 10 dilution. Clumps of spermatozoa formed by dilution of dry sperm with sea-water can be caused to disperse by the addition of ovarian extract.

When egg-sea-water, made by allowing the eggs of one female to stand in 25 cc. sea-water for half an hour, was used, exactly similar effects were obtained to those obtained with the ovarian extracts. Of course, dilution of this egg-sea-water reduced the effect, just as was the case with the ovarian extract. When, too, a drop of dry sperm was brought in contact with a drop of eggs and watched under the microscope, the sperm could be seen to move freely across the open spaces, and to gather about the eggs, but there was no sign whatsoever of a clumping reaction. These latter observations are in accord with, and to an extent provide an explanation of, the observations of Crozier (1922) that "during natural fecundation, however, no sperm-balls are formed. The thick, glutinous stream of spermatozoa passes under the girdle of the female, is somewhat diluted with sea-water by the tractive current, and emerges posteriorly in company with numerous large greenish eggs, about which, under the microscope, it can be seen that many sperms are gathered. But no real 'cluster formation' takes place."

Other substances that prevent clumping include the body juices of a mature male, body juices of a mature female, as was also noted by Crozier (1922), sublethal solutions of saponin in sea-water,[3] similar solutions of sodium taurocholate in sea-water, saturated and somewhat diluted solutions of trypsin in sea-water, and possibly by other substances. On the other hand, no prevention of clumping was obtained with acetone, ether, methyl alcohol, ethyl alcohol, adrenaline chloride (1 : 1000, 1 : 2000, or 1 : 5000), or carbon disulphide.

Discussion

It is obvious that in *Chiton tuberculatus*, the clumping reaction is different in every way from the agglutination reaction which has been described by Lillie (1913) for *Arbacia punctulata*, by Loeb (1914) for *Strongylocentrotus purpuratus* and *S. franciscanus*, by Just (1919) for *Echinarachnius parma*, and later (1929) for *Paracentrotus lividus* and *Echinus microtuberculatus*, by Carter (1932) for *Echinus esculentus*,

[3] The sources of the substances used for these tests were as follows:
Saponin: Eimer and Amend, New York, "A-61 Purified."
Sodium taurocholate: Eimer and Amend, New York, "A-61 Purified."
Trypsin: Eimer and Amend, New York, "A-61" "Pure."
Acetone: U. S. P., J. T. Baker Chemical Co., Lot No. 92237.
Adrenaline Chloride: Parke, Davis and Co., Detroit, Mich., U. S. A.
Ether, methyl alcohol, ethyl alcohol, and carbon disulphide: Usual laboratory supplies.

by Lillie (1913) for *Nereis limbata*, by Just (1915) for *Platynereis megalops*, and by other workers with other forms. With the agglutination reaction in these cases, the agglutination is between the heads of the spermatozoa, while the tails are apparently unaffected; the agglutination reaction is spontaneously reversible, cannot be repeated, and generally is produced only by substances secreted by eggs of the same species. With the clumping reaction in *Chiton tuberculatus*, on the other hand, the agglutination is between the tails while the heads are apparently utterly free and unaffected; the clumps, when once formed, persist indefinitely unless dispersed by means of the addition of certain substances, or by mechanical means. In the latter case, the clumps will reappear with an almost endless number of repetitions. The clumping forms as a perfectly natural and normal result of dilution with ordinary sea-water, and is, in addition, a phenomenon which can be prevented by means of body juices of the same form, egg and ovarian secretions and extracts, and certain lytic substances, such as saponin and sodium taurocholate.

With *Chiton tuberculatus*, it is unlikely that the clumping reaction, first described by Crozier, and described in detail herein, has any *direct* relation to the fertilization reaction as such. Instead, it is probably a mechanism by means of which masses of dry sperm may be transferred, in an intact manner, from the male to the mature female. This mass of dry sperm, thus transferred in an essentially intact condition, comes in contact with substances in the ctenidial channels of the female which destroy the substance which causes the tails to stick together and thus form clumps. The spermatozoa thus become freed from each other and are then able, by their own individual and utterly independent movements, to activate the all-environing eggs. The reason for transferring the spermatozoa to the female in an *intact* mass, however, might possibly reside in the need to preserve substances which might be essential for the actual fertilization reaction, and which might be rapidly lost from the spermatozoön in less concentrated suspensions.

The fact that these clumps can be dispersed by means of the proteolytic enzyme, trypsin, and by the lytic substances saponin and sodium taurocholate indicates that the clumping reaction in this form rests, fundamentally, upon the presence, on the outside surface of the spermatozoön tails at the time they are liberated from the testes, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by the above-mentioned substances. Since lytic substances, as suggested by Ponder (1930), might act by the destruction of the structure of proteins, a process which is also hastened

by trypsin, it is possible that the substance on the tails of the spermatozoa of dry sperm suspensions might be a sticky protein of some sort. On the other hand, Fieser (1937) has suggested that the hemolytic effect, of saponin at least, might be produced by a combination with cholesterol or lecithin of the cell membrane in a way such as to render the membrane permeable to hemoglobin, and evidence in partial support of this suggestion has been provided by Ransom (1901) in that he has shown with certainty that a combination of saponin and cholesterol is possible and that treatment of a saponin solution with cholesterol destroys its hemolytic activity. Popa (1927) has obtained evidence that the tails of the spermatozoa of *Arbacia punctulata* and *Nereis limbata* are enveloped by a large amount of lipoid substance.

Summary

1. When dry sperm of a mature male *Chiton tuberculatus* is introduced into the mantle cavity of a male or immature female and the discharged sperm is collected and examined immediately with a microscope, the spermatozoa will be seen to come together and form small clumps. *The point of attachment of these clumps is the tail*, while the heads remain perfectly free.

2. These clumps fuse readily to form either large spherical masses, or strands, which, in turn, soon fuse with other strands to form extensive networks. Such structures also form, readily and extensively, when the two drops are fused with the aid of a glass needle in a way such that the sperm suspension is distributed widely throughout the drop of sea water.

3. On the other hand, when extracts from the ovaries of ripe or spent females, egg-sea-water, body juices of a mature male, or of a mature female, or sublethal solutions of the lytic substances, saponin or sodium taurocholate, are used, *no clumping in any form occurs.* Instead, the spermatozoa in the advancing edge of the drop of dry sperm move freely, and the sperm mass progresses steadily until the far side of the drop of diluting fluid is reached.

4. These observations indicate that, in *Chiton tuberculatus*, the clumping reaction rests fundamentally upon the presence, on the outside surface of the spermatozoön tails, of a substance which (1) is distinctly sticky in nature, and which (2) can be dissolved or destroyed by certain substances.

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THE LUMINESCENCE OF A NEMERTEAN, EMPLECTONEMA KANDAI, KATO

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Introduction

A great many species in the five phyla, Plathelminthes, Nemertea, Trochelminthes, Nemathelminthes and Chaetognatha, are closely allied to one another. Among these species, no luminous form has been previously recognized. I found, however, a number of luminous nemerteans, when I visited the Marine Biological Station of the Tohoku Imperial University at Asamusi, Aomori, Japan in the summer of 1936.

These nemerteans had coiled up on *Chelyosoma*, which were collected from the bottom of Aomori Bay between Natutomari and Aburame at a depth of about 35–40 meters, and were placed in the laboratory for study. They were identified by Koziro Kato (paper in preparation) as *Emplectonema kandai* sp. nov.

It is an extraordinary fact that among so large a number of species of the five phyla, only one is found to be luminous. *Emplectonema* is a genus of the nemerteans which is widely distributed in America, the Atlantic and Pacific Oceans, the Mediterranean Sea, the White Sea and Japan. It may be expected, therefore, that more luminous species of the same genus, at least, will be observed somewhere in the future. I made some experiments on *Emplectonema kandai* during the three summers of 1936–38 at the Station mentioned above. The results are given in the present paper.

I wish to express my sincere appreciation of the facilities afforded me there by Professors S. Hatai (1937) and S. Hozawa (1938), Directors of the Laboratory. I would also like to acknowledge my indebtedness to Messrs. N. Abe, K. Atoda and K. Kato, without whose aid this paper would not have been completed.

Material

As already stated, these luminous nemerteans coiled up on *Chelyosoma*. It is necessary, therefore, to collect the latter, which are dredged (by three fishermen) from the bottom of Aomori Bay, about

[1] A preliminary note in Japanese was published in the *Rigakukai*, **35** (1937): 5–11.

15 km. off the Station. But the nemerteans are not abundant. Only six or seven individuals at best, or sometimes only one or two individuals, are obtained on about two hundred *Chelyosoma*, which are collected by the fishermen as the result of one day's work.

The nemerteans are reddish orange in color. They have many eyes. They vary in length, from 53–115 cm., and are about 0.5–0.7 mm. in diameter, when they are stretched. I found one individual 10 cm. long, but one so short is extremely rare. The female animal is readily distinguished during the summer season because it is full of eggs or enlarged gonads, but I could not distinguish the males. The animals coil up on the wall of a large vat of running sea water or on the bottom, attached by the slime which is abundantly secreted from the surface of its body (Fig. 1). They remain there quietly for two months or more, if they are not disturbed.

FIG. 1. The living and coiled whole *Emplectonema kandai* about 115 cm. long. About natural size. (Photographed by N. Abe at my request.)

The animals flash brilliantly only on stimulation. The stimulus may be mechanical, chemical, thermal or electrical. The light may appear on all parts of the body, but it disappears in one or two seconds. It is whitish green in color.

Mechanical Stimuli

The animal flashes when a glass rod or a finger is gently touched to the surface or surfaces of the coiled body. The light does not spread very far from the place or places of the contact, and lasts for only one or two seconds. Its intensity varies, depending on the strength of the contact. I thought at first that some luminous material was thrown into the sea water mixed in a slime discharged from the surface of the animal body, but this observation turned out to be incorrect.

If the coiled animal is strongly rubbed between the fingers, a brilliant light appears, but it is not observed that any luminous material comes off which adheres to the fingers. If the animal is suddenly extended, without being broken, between two hands, the

head in one hand and the tail in the other, the brilliant light also appears through the whole surface of the long body except the tip of the head. This luminescence is a most beautiful sight.

Chemical and Osmotic Stimuli

If the sea water containing the nemertean is acidified with a very dilute HCl or acetic acid, the animal gives a bright light. The acid should not be too strong, or it will kill the animal too quickly. The dead or dying nemerteans produce light continuously, until all the luminous material is probably exhausted. The addition of dilute NaOH or NH_4OH to the sea water produces the same effect, although it precipitates the Ca and Mg of the sea water.

The best way to test the luminescence of the animal is to add dilute H_2O_2 to the sea water. This action is not injurious and is reversible.

If 1 to 2 cc. each of $\frac{M}{2}$ NaCl, $\frac{M}{2}$ KCl, $\frac{M}{2}$ NH_4Cl, $\frac{M}{2}$ $MgCl_2$, $\frac{M}{2}$ $MgSO_4$, $\frac{M}{2}$ Na_2SO_4, or $\frac{M}{2}$ $(NH_4)_2SO_4$ solution are added to 100 cc. of sea water which contains a nemertean, no luminescence is observed. In 0.5 cc. $\frac{M}{2}$ $CaCl_2$ plus 100 cc. sea water, however, the animal begins to flash occasionally after about 10 minutes. The intervals of its flashing become quite regular after about 20 minutes, resembling those of a firefly. Besides these flashes, there is a very faint and continuous light in other parts of the body. If this treated animal is removed to normal sea water after about 40 minutes, it lives normally. In 1 or 2 cc. $\frac{M}{2}$ $CaCl_2$ plus 100 cc. sea water, the intervals of the flashing are very slow and somewhat irregular.

In pure $\frac{M}{2}$ KCl, $\frac{M}{2}$ $CaCl_2$ or $\frac{M}{2}$ Na_2SO_4 solution, practically isotonic with sea water, the animal gives a bright light. But if the animal is kept too long in the solution, it will be killed. A mass of slime is secreted into each solution, but no luminous material is observed in it. In pure $\frac{M}{2}$ $MgSO_4$ solution, the animal flashes after about 8 minutes. In pure $\frac{M}{2}$ NH_4Cl solution, it gives a faint light after about 20 minutes. It would seem that K, Ca, Na, Mg, or NH_4 ions cause the luminescence of the animal.

In pure $\frac{M}{2}$ NaCl or $\frac{M}{2}$ $MgCl_2$ solution, isotonic with sea water, the

animal gives no light. In pure $\frac{M}{2}$ $(NH_4)_2SO_4$ solution, also, no luminescence appears. In these cases, no cation, Na, Mg or NH_4, seems to cause any light whatever. It is a little difficult to decide from these experiments whether cation or anion stimulates the animal to become luminous since no luminescence is observed in NaCl, $MgCl_2$ or $(NH_4)_2SO_4$ solution, whereas some luminescence occurs in Na_2SO_4, $MgSO_4$ or NH_4Cl solution.

If crystals of NaCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MgSO_4$, Na_2SO_4, or $(NH_4)_2SO_4$ are added to 20 cc. of sea water, which contains the nemertean, a brilliant light is always observed. If a large amount of salt is used, the light is continuous and fades gradually, due to the death of the animal. On addition of fresh water or distilled water to the sea water, a bright light appears also. Saponin acts in the same way. The increase or decrease of osmotic pressure plays, of course, a distinct rôle in each case.

Temperature and Electrical Stimuli

If the sea water at 20° C., which contains the animal, is heated to 32–33° C., or is cooled to about 1° C., the animal produces light. With induced currents, the animal also gives light.

Luciferin and Luciferase

If the animals are placed on a heavy blotting paper, they give a bright light immediately. When they are dried over P_2O_5, light is still observed during the drying and the dried, dead animals give light when again moistened with water. When these moistened ones are dried again over P_2O_5, however, they produce no more light on being moistened again with water. The animal is slender and all parts of the body are covered by a simple, thin epithelium, where the luminous cells are located, as are the cells of other luminous animals. Evidently the luminous cells of the nemertean are not large, as the cross-section of the animal indicates (Fig. 2). This may explain why the amount of luminous material secreted by the cells is comparatively small.

The existence of luciferin and luciferase cannot be demonstrated in the usual way with either the fresh or the dried animals, which are ground with sand in a mortar and are extracted with hot or cold water. Methyl or ethyl alcohol extracts of the fresh and dried nemerteans also give no light with cold or hot water extracts. The cold water extract of the nemertean gives no light with *Cypridina luciferin*, nor does the hot water extract of the nemertean give light with *Cypridina luciferase*.

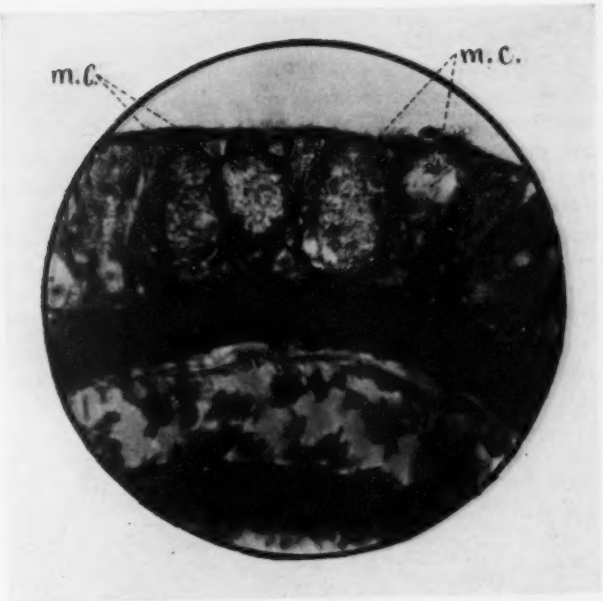
Potassium Cyanide

Since the luciferin-luciferase reaction cannot be demonstrated, the question may be asked: Is not the luminescence of the nemertean due to the symbiosis of luminous bacteria? Pierantoni (1918) holds that the light of all animals is due to symbiotic luminous bacteria. I did not attempt to raise luminous bacteria from the nemertean on an artificial culture medium, but studied the effect of KCN. According to Harvey (1921), the light of marine luminous bacteria disappears in 4 minutes, if they are treated with $\frac{M}{20}$ KCN solution, namely 0.325 per cent solution, and in 6 minutes, if treated with $\frac{M}{40}$ KCN or 0.162 per cent solution. He also shows that the light of an emulsion of the luminous organ of a fish, *Photoblepharon*, which is suspected to be symbiotic, disappears in about 20 minutes, if it is treated with 0.25 per cent KCN solution, and in about 30 minutes, if treated with 0.125 per cent KCN solution. I have found that the nemertean gives light immediately and that the light continues for about 110 minutes, if 1 cc. of the aqueous solution of $\frac{M}{2}$ KCN is added to 10 cc. of sea water which contains the animal. The animal begins to give light after 50 minutes and continues for about 140 minutes, if 0.5 cc. of $\frac{M}{2}$ KCN solution is added to 10 cc. of sea water. In both cases, the animal dies, not from dilution of sea water, but from KCN, since the worm can live indefinitely without evident injury in 100 cc. of sea water plus 20 cc. of distilled water.

These facts indicate that KCN, which inhibits most cell oxidations instantly, has very little effect on the luminescence of the nemertean. They would seem, also, to show that the light of the animal is not of bacterial origin. The failure to prove the presence of luciferin and luciferase in the animal does not necessarily indicate the symbiosis of luminous bacteria. On the contrary, I believe that luminescence in this nemertean arises from a chemical luminous material secreted in its luminous cells.

FIG. 2. Microscopic photograph of portion of a transverse section of the body surface of *Emplectonema kandai*, showing mainly the mucin-secreting cells (*m. c.*). About × 500. (Section prepared by K. Atoda at my request.)

FIG. 3. Microscopic photograph of portion of a transverse section of the body surface of *Emplectonema kandai*, showing some light-producing cells (*l. c.*) and mucin-secreting cells (*m. c.*). About × 500. (Section prepared by K. Atoda at my request.)



FIGS. 2 and 3.

Histology

I have studied the transverse and longitudinal sections of this worm, which had been kindly prepared by K. Atoda and K. Kato. The epithelium of the worm is very simple, though it is comparatively wide. In general, there appear in the epithelium, two kinds of glandular cells which stain with Delafied's haemotoxylin and eosin. Those which stain blue with haemotoxylin are large and open through the cuticle of the epithelium (Fig. 2): They are apparently the mucin-secreting

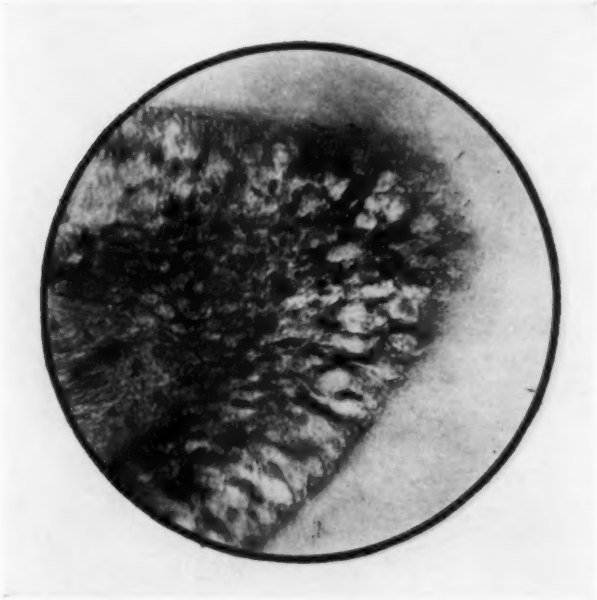


FIG. 4. Microscopic photograph of portion of a longitudinal section of the head-tip surface of *Emplectonema kandai*, where no light cells show. About × 500. (Section prepared by K. Kato at my request.)

cells. In some preparations, however, a great many cells are almost devoid of slime, which was probably discharged while the worm was being narcotized with menthol.

The cells staining with eosin appear to consist of two types, although this is not always evident. Those of one type, which stain red with eosin, though not very deeply, show a small nucleus at the base, are elongate and open through the cuticle. They are filled with granules. These cells are most common throughout all preparations

studied. The cells of the other type are especially evident when Mallory's stain is used. They stain deeply with eosin. Under a high power of the microscope they are seen to contain fine granules and in some cells their content is homogeneous. I assume that these cells are merely the young, unripe ones of the second type.

I believe that all the cells which stain in eosin are the light cells of the worm (Fig. 3). It is interesting to note that the tip of the head of the worm, where no light appears, as already stated, shows none of the eosinophil cells at all (Fig. 4.) In the head or anterior part farther from the tip, however, there appear some cells, which stain deeply with eosin and also contain the granules typical of the light cells. The number of such cells increases gradually towards the middle of the worm.

As I have already stated, the luminous material of the worm has not been observed to separate from the cuticle. But this does not mean that the glandular structure of the ducts has no opening or pore in the cuticle. On the contrary, all the ducts appear to have openings, as the sections show. The luminous secretion should be very small, however, as the light cells are also small, and the light production may take place at the instant of discharge, or the light-giving action may take place in the cells before the substances reach the openings of the ducts in the cuticle.

This work was aided by a grant from the Foundation for the Promotion of Scientific and Industrial Research of Japan.

Summary

The luminescence of a marine nemertean worm, *Emplectonema kandai*, living on *Chelyosoma*, is described. Light appears from the whole of the body, except a small region at the head end, in response to mechanical, chemical, thermal or osmotic stimulation. The effect of salts has been studied.

The photogenic cells are in the epithelium, stain with eosin, and appear to have openings in the cuticle, but no extracellular luminous secretion could be demonstrated. Histological sections are figured.

Luciferin and luciferase could not be demonstrated, but since KCN does not inhibit luminescence, the origin of the light is thought to be the gland cells of the worm and not symbiotic bacteria.

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ABSENCE OF THE EPITHELIAL HYPOPHYSIS IN A FETAL DOGFISH ASSOCIATED WITH ABNORMALITIES OF THE HEAD AND OF PIGMENTATION

DON WAYNE FAWCETT

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The specimen herein described is an albino fetus of the spiny dogfish (*Squalus acanthias*) which presents malformations of the head including cyclopia and astomia. Associated with these is the very rare anomaly—absence of the epithelial hypophysis. It is well known that there are abnormalities of the pituitary in human anencephalic fetuses, but these involve absence of the neural lobe. Covell (1927), in making a quantitative study of such abnormal human fetuses, reviewed the literature and reported personal observations comprising in all nearly a hundred cases. He concluded that an hypophysis is always present, although the lobus nervosus is lacking in the majority of specimens. The pars anterior he found constitutes most of the gland volume and in some cases the total volume. He mentions no case of absence of the anterior lobe. The only instance hitherto described of spontaneously occurring absence of the anterior pituitary, in any animal, is the case reported by Evelyn Holt (1921) of absence of the pars buccalis in a 40-mm. pig.

The study of the present specimen of an elasmobranch fetus has been undertaken not only on account of its rarity but because of the evidence of interdependence in development of the separate lobes of the pituitary gland and the effect of absence of the oral components of the gland on pigmentation.

Material and Methods

The fetus was discovered among a great number of normal *Squalus* pups, at the David Richardson Laboratory, Bailey Island, Maine. Because its abnormalities escaped notice when it was removed from the uterus, normal littermates are not available for study as controls. Two normal pups, from other uteri, but of a comparable stage of development, were selected, instead, as controls. The three fetuses were fixed in 7 per cent formalin. The heads were subsequently imbedded in celloidin, cut serially in 35 μ sections and stained with haematoxylin and eosin.

Description

The specimen is silvery white with the exception of the tip of the dorsal fin and the tip of the tail where in each case a small area is deep grey, approaching in intensity the color of a normal pup. The otherwise translucent skin is faintly clouded with light grey over the dorsum of the body, suggesting that the albinism is not due to complete absence of pigmentation. Aside from this general albinism, the obvious external deviations from the normal are limited to the region rostral to the first gill slits; the rest of the body is of normal configuration. The eyes give the appearance of having been drawn from their usual lateral position ventrally and medially to the midline where they are fused into a single dumbbell-shaped eye with two discrete lenses. There are no external nares; only a midventral prominence indicates where the olfactory bulbs have coalesced. The mouth is represented by a dimple-like depression and a narrow fold formed by a shallow, rostrally directed invagination of the integument (Fig. 1). In addition to these abnormalities of the head, it is noted that the yolk sac is exceptionally large as compared to that of normal fetuses.

Examination of the sections reveals radical departures from the normal structure of the cartilaginous cranium, a description of which requires frequent reference to the normal processes of development in order to understand and interpret them correctly. The base of the elasmobranch chondrocranium normally develops from two pairs of cartilaginous bars. The posterior or parachordal cartilages constitute the caudal part of the basis cranii. In the anterior or prechordal region, the cranial floor develops from paired trabeculae cranii whose rostral ends fuse in the midline to form the interorbital plate and the more anterior rostral plate. Between the diverging posterior ends of the trabeculae and the anterior edge of the basal plate is a median space, the fenestra hypophyseos, through which the hypophysis and carotid arteries gain entrance to the cranial cavity. The original hypophyseal connection with the oral cavity is gradually obliterated by centripetal growth of these cartilages. In the present malformed cyclopean fetus the abnormalities of the chondrocranium itself are limited almost entirely to the prechordal region. Cyclopean teratogenesis is generally believed to occur very early in embryonic development of the eyes and is attributed to a local arrest of growth ventrally, with fusion of the elements of the primary optic vesicles and normal growth of dorsal parts. It is commonly thought that the cyclopean eye obstructs the path of forward growth of the cranial trabeculae—and this appears to have occurred in the present specimen. As one can observe in Figs. 7 and 8, there is an amorphous horizontal plate

of cartilage which overlaps the back of the posterior edge of the eye and extends caudally to the level of the spiracle. Even though this cartilage does not unite with the basal plate at any point, it seems probable that it represents a fusion of the trabeculae in a somewhat abortive attempt to grow forward past the obstructing eye. This cartilage does not approach the basal plate caudally, as might be expected, because of the interposition of jaw elements. The primordium of the mandibular arch normally takes the form of an inverted *U* at each corner of the mouth. In this specimen the dorsal parts of the head have unfolded normally, carrying lateralward the pterygoquadrate limb of the *U*-shaped cartilages while at the same time arrest of growth in the midventral line has caused the mandibular limb (Meckel's cartilages) to be crowded against the median basihyoid plate. Subsequently there appears to have been more or less fusion of these cartilages such that, at the level of Fig. 9, they constitute a single plate of cartilage in which the pterygoquadrate components are represented most laterally, Meckel's cartilages next, and the basihyoid cartilage in the middle. The homologies ascribed to the abnormal cartilaginous elements seem justified because the conspicuous adductor mandibuli muscle complex which normally surrounds the angle of the jaw between pterygoquadrate and mandibular elements (Fig. 9-*a*) is oriented with respect to the abnormal fused mass of cartilage precisely as would be expected if the above explanation were correct (Fig. 9).

The brain is for the most part normal save for minor readjustments in relation to the misplaced olfactory bulbs and retinae. In the normal dogfish the floor of the diencephalon bulges ventrocaudally forming the infundibulum which consists of two hollow oval lobes—the inferior lobes. A long tongue of glandular tissue consisting of the pars distalis and pars medialis of the pituitary extends anteriorly from the pars intermedia and may be seen lying in the groove between the inferior lobes (Fig. 5-*a*). At the level of the emergence of the oculomotor nerves the inferior lobes of the infundibulum are normally continuous with a thin-walled vascular outgrowth, the saccus vasculosus (Fig. 6-*a*), which is connected posteroventrally with the glandular intermediate lobe. At this point, in the normal animal, nervous tissue from the thickened floor of the saccus vasculosus is commingled with

PLATE I

FIG. 1. Ventral view of the abnormal *Squalus acanthias* fetus (natural size). The yolk-sac has been removed.

FIG. 2. Ventral view of a normal dogfish fetus.

FIG. 3. Dorsal view of the abnormal albino dogfish fetus.

FIG. 4. Dorsal view of a normal dogfish fetus.

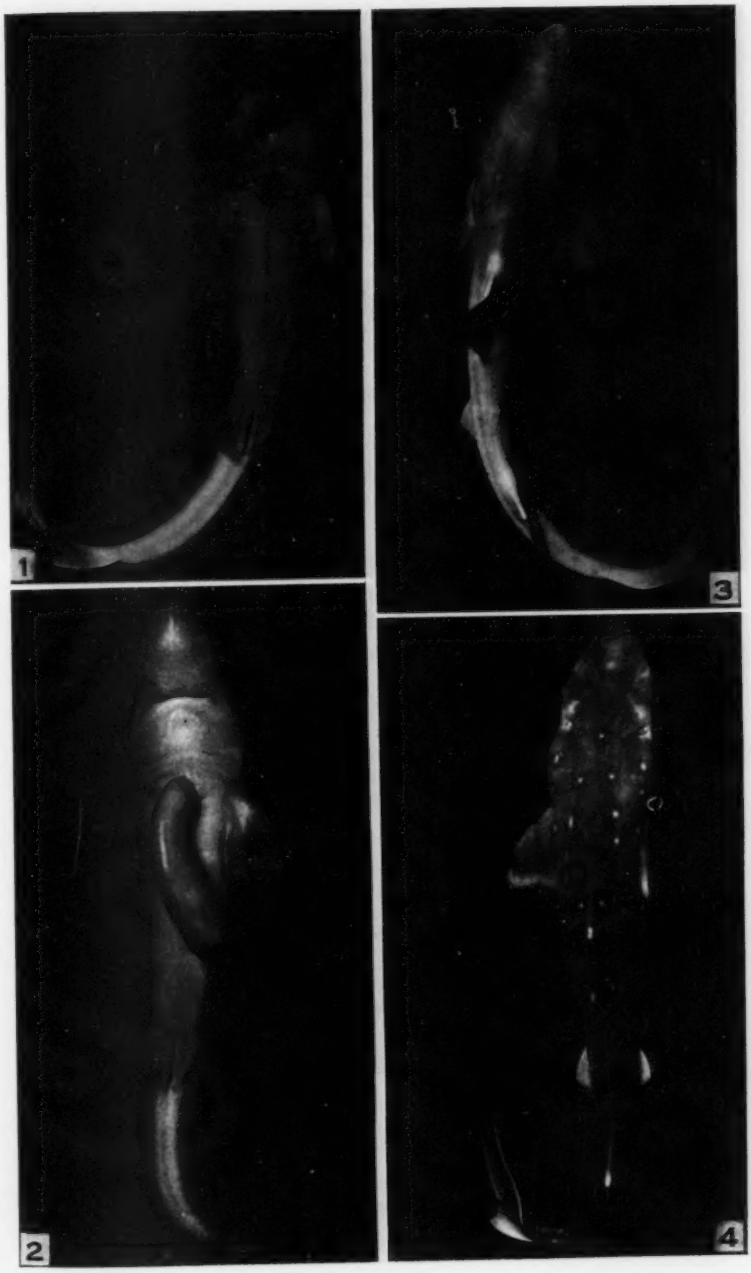

PLATE I

the glandular cords of the pars intermedia. The nervous tissue around this area of contact is usually thought to be the functional pars neuralis. The pars neuralis undergoes but little differentiation, the cells around

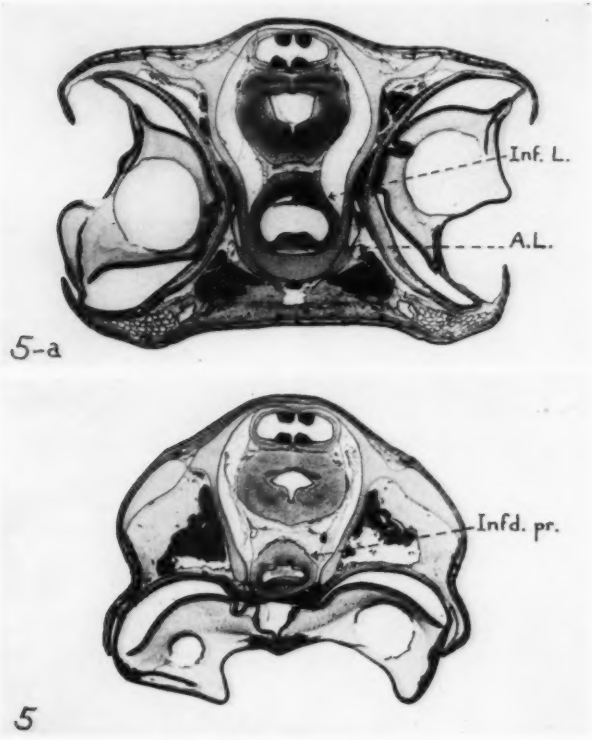

PLATE I

FIG. 5-*a*. A section of the head of a normal fetus showing the pars distalis of the anterior lobe (*A.L.*), lying in the groove between the inferior lobes of the infundibulum (*Inf.L.*). Photomicrograph (hematoxylin and eosin; × 4).

FIG. 5. A corresponding section of the head of the anomalous fetus showing no anterior lobe and a deformed infundibular process (*Infd. pr.*) not supported beneath by an interorbital plate of cartilage. Photomicrograph; × 4.

the infundibular lumen retaining much of the character of the embryonic ependymal layer (Butcher, 1936).

In the present abnormal specimen, on the contrary, the infundibulum extends posteriorly in a long conical process which, due to

absence of the anterior part of the cranial floor, is for some distance not supported beneath by cartilage but traverses the groove between the two dorsal convexities of the malformed eye (Fig. 5). This attenuated infundibulum becomes gradually more slender as it passes out of the cranial cavity beneath the anterior edge of the basal plate. The basal plate dorsally, the pterygoquadrate and mandibular rudiments laterally, and a ventral plate of cartilage (possibly the basihyoid) are all partially fused about a tubular space in which the narrow infundibulum is lodged (Fig. 7). Caudal to this point these fused cartilages separate from the base of the chondrocranium providing much more space for the infundibular process (Fig. 8). In consequence of this, the infundibulum expands at its caudal tip into a bulbous enlargement which ends blindly at the level of the spiracle in contact with the blind end of the entodermal pharynx (Fig. 9). The infundibular recess which extends from the third ventricle into the infundibular stalk is nearly obliterated at its narrowest point but finally terminates in a conspicuous cavity within the terminal bulbous enlargement of the neurohypophysis. At no place does the infundibular process show any tendency to differentiate into a saccus vasculosus.

The most striking abnormality of the pituitary is the total absence of the epithelial portions of the gland. No trace can be found of the parts of the pituitary deriving from the embryonic stomodeum—pars distalis, pars medialis, pars intermedia, and pars ventralis.

Discussion

The pigmentary deficiencies, the absence of all buccal components of the pituitary, and the aberrant infundibular process exhibited by this anomalous fetus are of interest in connection with the findings of P. E. Smith in albino tadpoles produced by early ablation of the pars buccalis of the hypophysis. It was shown by him that atypically placed buccal epithelium would induce hypertrophy of adjacent nervous tissue whereas in complete absence of the buccal hypophysis, the neural lobe did not attain its normal size, shape, or histological development. Evelyn Holt's 40-mm. pig, while lacking entirely the oral portion of the hypophysis, is described as possessing a pars neuralis "normal in position, extent, and structure." This, she points out, is contradictory to Smith's findings but it is noteworthy that her specimen is from a relatively early stage of development. If the pig fetus had had an opportunity to continue its intrauterine life, the further growth of the pars neuralis might well have been retarded or modified.

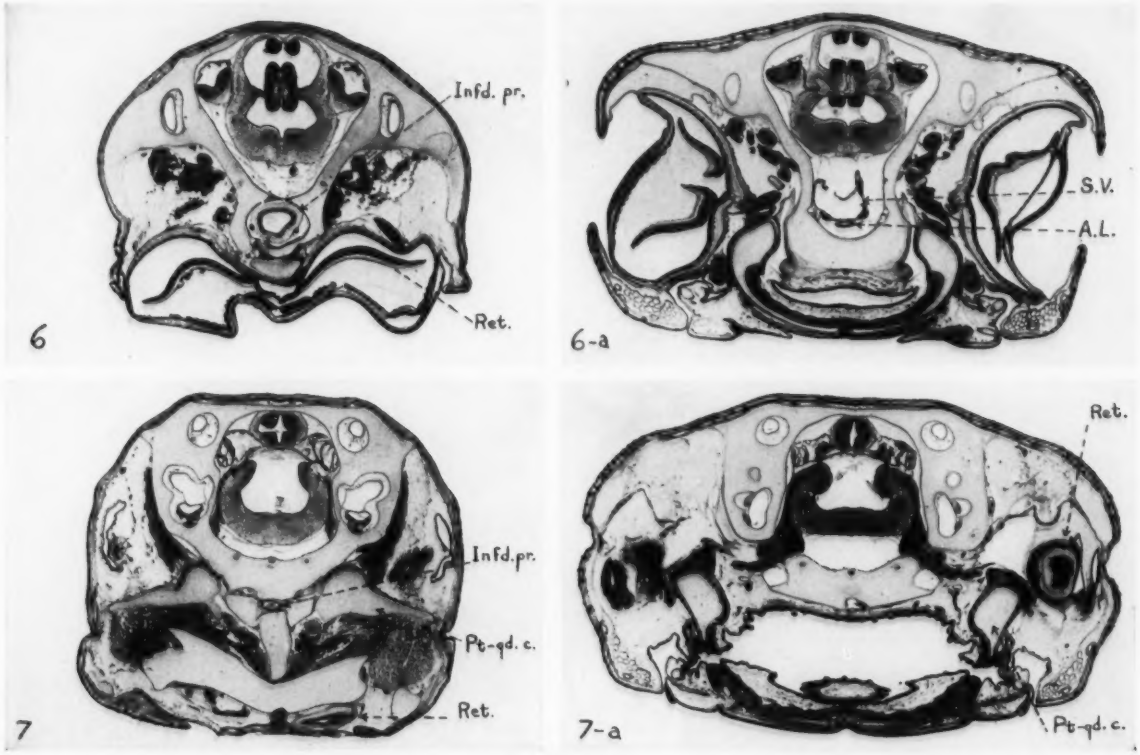

PLATE II

FIG. 6. Section of the head of the abnormal specimen showing the undifferentiated caudal extension of the infundibular process (*Infd. pr.*).

FIG. 6-*a*. Section at a corresponding level of the head of a normal control showing a saccus vasculosus (*S.V.*), and anterior lobe (*A.L.*) of the pituitary. Photomicrographs × 4.

FIG. 7. Photomicrograph of a section of the abnormal fetus at the level of the posterior edge of the retina (*Ret.*) showing the constricted region of the infundibular process (*Infd. pr.*) lodged between closely surrounding cartilages. Pterygoquadrate cartilage (*Pt-qd. c.*).

FIG. 7-*a*. Photomicrograph of a corresponding section of the normal control showing no part of the hypophysis present at this level.

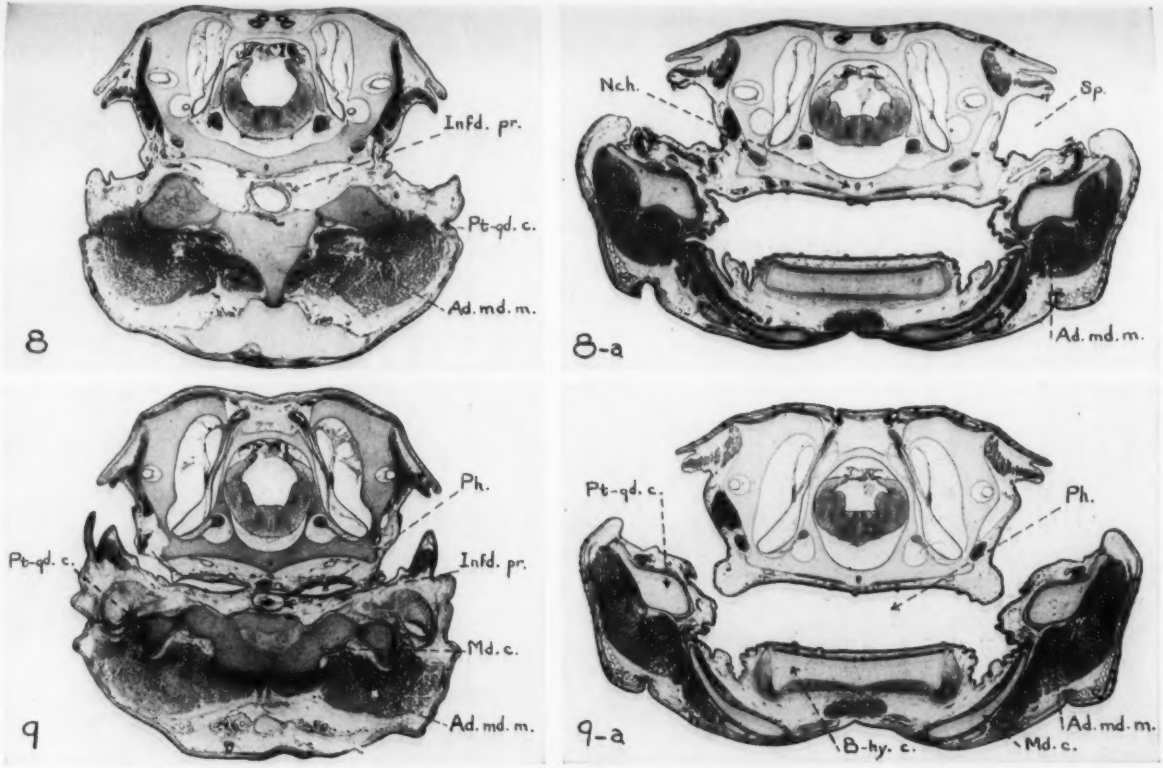

PLATE III

FIG. 8. Photomicrograph (× 4) of a section of the malformed specimen at the level of the spiracle (*Sp.*) showing the terminal bulbous enlargement of the infundibular process (*Infd. pr.*). The homologue of the pterygoquadrate cartilage (*Pt-qd. c.*) is associated with the conspicuous adductor mandibuli muscle (*Ad. md. m.*).

FIG. 8-*a*. A corresponding section of the normal control. At this level there is no part of the hypophysis.

FIG. 9. Photomicrograph of a section of the anomalous fetus at the level of the posterior edge of the spiracle showing the caudal end of the infundibular process in contact with the blind anterior end of the pharynx (*Ph.*). Mandibular cartilage (*Md. c.*).

FIG. 9-*a*. A corresponding section of a normal fetus. Pterygoquadrate cartilage (*Pt-qd. c.*). Mandibular cartilage (*Md. c.*). Basihyoid cartilage (*B-hy. c.*).

Furthermore, the interdependence in development of one part of the pituitary upon another may be quite different in mammals than in amphibia and fishes. The present case of pituitary agenesis in a lower vertebrate seems to bear out Smith's observations, for here, as in his tadpoles, in the total absence of the buccal ectoderm, the pars neuralis has not attained its normal shape nor has it undergone its typical differentiation into a saccus vasculosus.

Tadpoles in which the buccal hypophysis has been removed at an early stage of development display albinism in which the epidermal melanophores are diminished in number and pigment content besides remaining in a persistent state of contraction. These conditions in the tadpole are closely paralleled by this albino dogfish fetus in which the melanophores are less numerous, contracted, and noticeably withdrawn from the surface. Lundstrom and Bard, in a study of the effects of ablation of various parts of the brain of the dogfish (*Mustelis canis*), first discovered the hypophyseal control of the cutaneous pigmentation in the elasmobranch fishes. They found that removal of the neuro-intermediate lobe invariably resulted in pallor of the skin. The present anomalous specimen constitutes an interesting confirmation of their work. Evidently there has been a spontaneous suppression of the oral hypophysis equivalent to actual experimental ablation. Because of the intermingling of the elements of the pars neuralis and pars intermedia of the dogfish pituitary, it has so far been impossible to accomplish a complete operative separation of these two parts. The presence of the neuro-hypophysis in the present specimen, but the total absence of the oral pituitary (including the pars intermedia) indicates that the humoral agent affecting pigmentation is a derivative of the oral components of the gland. Presumably in that portion of the gland referred to as the neuro-intermediate lobe it is the buccal elements that are responsible for the chromatophore-expanding factor.

Observations in many cases of human anencephaly (Covell, 1927) make it apparent that aberrant formative processes involving defective closure in the dorsal midline may result in agenesis of the neural lobe of the pituitary. Cyclopia and astomia are occasionally found together in human monsters. It appears from the present observations that, in the dogfish, anomalous development in the ventral midline with imperfect separation of symmetrical parts and consequent cyclopia and astomia may result in agenesis of the oral hypophysis.

Summary

1. An anomalous fetus of the spiny dogfish (*Squalus acanthias*) is described in which there are malformations of the head comprising cyclopia, astomia, and abnormalities of the hypophysis.

2. The abnormalities of the hyophysis involve:—

(*a*) The total absence of the oral components of the gland, and

(*b*) A neural lobe which is deformed and possesses no saccus vasculosus. The conclusion is drawn that the neural lobe has not undergone normal differentiation because it has been deprived of its usual association with the buccal hypophysis.

3. The specimen is albino, displaying a diminished number of chromatophores in a state of persistent contraction. This finding indicates that the melanophore-controlling principle in the dogfish is a derivative of the buccal components of the pituitary.

4. Only one other instance of total spontaneous suppression of the oral hypophysis is described in the literature, namely, in a pig fetus (Holt, 1921). In human fetuses anencephaly occurs not infrequently but is associated with suppression of the neuro-hypophysis instead of with the adenohypophysis.

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VARIATIONS OF COLOR PATTERN IN HYBRIDS OF THE GOLDFISH, CARASSIUS AURATUS

H. B. GOODRICH AND PRISCILLA L. ANDERSON [1]

(From the Department of Biology, Wesleyan University)

This paper gives an account not only of the differences between fish arising from the same genetic cross but also of the variations of color pattern taking place during the life of individual fish.

The cross between the common goldfish and the transparent shubunkin which are both varieties of the species *Carassius auratus* was first subjected to genetic analysis by Berndt (1925 and 1928) and Chen (1925 and 1928). The results indicated that the two parental types are genetically distinguishable by a single gene difference. The formulae as denoted by Chen are: common goldfish TT, the transparent shubunkin $T'T'$, and the hybrid TT'. This hybrid is known to the fanciers as the calico shubunkin. The common goldfish, which is quite brown or black during youth, changes to the familiar orange or red type by destruction of part or nearly all of its melanophores (Berndt, 1925; Goodrich and Hansen, 1931). This type also carries at least two layers of reflecting tissue, one beneath the scale layer and the other backing each individual scale. The transparent shubunkin has lost most of the chromatophores (both melanophores and xanthophores) and also most of the reflecting tissue. The heterozygous type, or calico fish, shows great variability in the distribution of both melanophores and xanthophores and there is no bilateral symmetry of pattern. A deep abdominal layer of reflecting tissue is present and a few scales are also backed with the tissue. For full details, papers by Chen (1928) or Goodrich and Hansen (1931) may be consulted.

Goodrich and Hansen (1931) made a detailed comparative study of the history of the melanophores of the three phenotypes covering the first eight weeks after hatching during which period the fish grew from 4.5 mm. to about 33 mm. in length. It was found that the history of the three types was similar for the first week (to 9 mm.) showing a uniform rate of multiplication of the chromatophores. After this the three types diverged. The normal goldfish showed a very rapid

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and uniform increase in number of chromatophores. In the transparent shubunkin the melanophores began to disintegrate until nearly all were destroyed. The hybrid, however, was found to be highly variable, showing great diversity between individuals. New cells appeared; others were destroyed. It gave the impression of a conflict between the cell proliferation and cell destruction.

Material and Methods

This paper continues the observations on the melanophore pattern of the heterozygous type beginning where the previous study was discontinued. The work was begun during the summer of 1937 with fish varying from 23 to 36 mm. in length (tip of mouth to base of caudal fin). The fish were chiefly obtained from the Grassyfork Fisheries of Martinsville, Indiana, to which institution we are greatly indebted. The hybrid fish were obtained directly from the hatchery which raises them regularly for the market. Records were made by photographing one side of the fish at intervals of approximately one month, but the periods were lengthened to longer intervals during the last six months. Ten of the fish are still under observation at this time, one year and six months after the start of the work. They vary from 47 to 58 mm. in length. All others that were started died. Anaesthetization, necessary for photography, proves to be fatal in some cases.

The individuals differ markedly from each other. For purposes of description two types, *A* and *B*, may be recognized, but it should be understood that there are intermediate gradations. Type *A* shows a relatively uniform distribution of melanophores on the dorsal half of the body and extending variably below the lateral line (Figs. 1 and 2). In Type *B*, the distribution of melanophores is much more uneven. They tend to be aggregated in clusters (Figs. 4 and 5). Xanthophores are present in both types and are unevenly distributed, but are not studied in this paper as it is very difficult to distinguish and identify the individual cells.

History of Color Patterns

Type A

It is possible with these fish to enumerate and reidentify from time to time all cells of large areas on the photographed side of the body. Except in the cases where wholesale destruction of melanophores occurs, it is found that few cells are lost and that individual cells have long life. An example may be taken (our fish number *MG*-3) on which 907 cells were enumerated and located on the side of the body (see

Table I for this and other references to cell counts). The first photograph was taken August 2, 1937 and the last February 17, 1939 making a total series of 18 photographs. During this time, 50 of the 907 cells disappeared and three new ones appeared. Figure 1 is the photograph

TABLE I

This table gives records of photographs of four of the fish studied. The dates are accurate only for *MG*-3 as it was not always possible to take all photographs on the same day.

	MG-3 Calico Type *A* 907 cells		*MG*-4 Calico Type *B* 97 cells, 2 cl.		*MG*-15 Transparent 21 cells		*MG*-16 Calico Type *A* 613 cells	
	D	*A*	*D*	*A*	*D*	*A*	*D*	*A*
July 17, 1937	0	0	0	0(4)				
Aug. 2	0	0(1)	9	1 cl	0	0	0	0
Aug. 26	1	0	6	2 1 cl	0	0	6	0
Sept. 20	5	0	2	0	0	0	1	0
Oct. 18	5	0	0	0	1	0	0	1 cl
Nov. 16	5	0	7	0	1	0	(6)	0
Dec. 15	4	0	4	1 cl	0	0		0
Jan. 26, 1938	2	0	3	4 cl	0	0		0
Feb. 23	4	0	7	2 cl	0	0		0
Mar. 28	1	0	1	3 cl	0	0		0
May 2	4	1	1	0	1	0		0
June 7	9	1	1	0	1	0	(7)	0
July 16	2	1	0	0	0	0		0
Aug. 5	2	0	1	0	0	0		0
Sept. 13	–	–	8	0	0	0		0
Oct. 12	4	0	1	0	0	0		0
Nov. 14	0	0	0	0	0	0		0
Jan. 4, 1939	0	0(2)	0	0(5)	0	0		0
Feb. 17	2	0	0	0	0	0		0
Totals	50	3	51	2 12 cl	4	0	All	1 cl

D—number of cells that disappeared since preceding photograph.
A—number of new cells appearing since preceding photograph.
cl—cell cluster or spot.
(1), (2), (4), (5) indicate pictures reproduced in Figs. 1, 2, 4, and 5.
(6)—time of beginning of wholesale destruction of melanophores.
(7)—time at which all melanophores were destroyed.

taken August 2, 1937 and Fig. 2 that of January 4, 1939. The dotted lines outline arbitrarily delimited areas marked on the prints to facilitate the counting and identification of cells. The small circles indicate the former location of cells that have disappeared. Figure 2 is taken at a lower magnification than Fig. 1 and fish had grown from 26 mm.

to 58 mm. in length (exclusive of caudal fin). Figure 3, however, shows the rectangular area of Fig. 2 raised to the same magnification as Fig. 1.

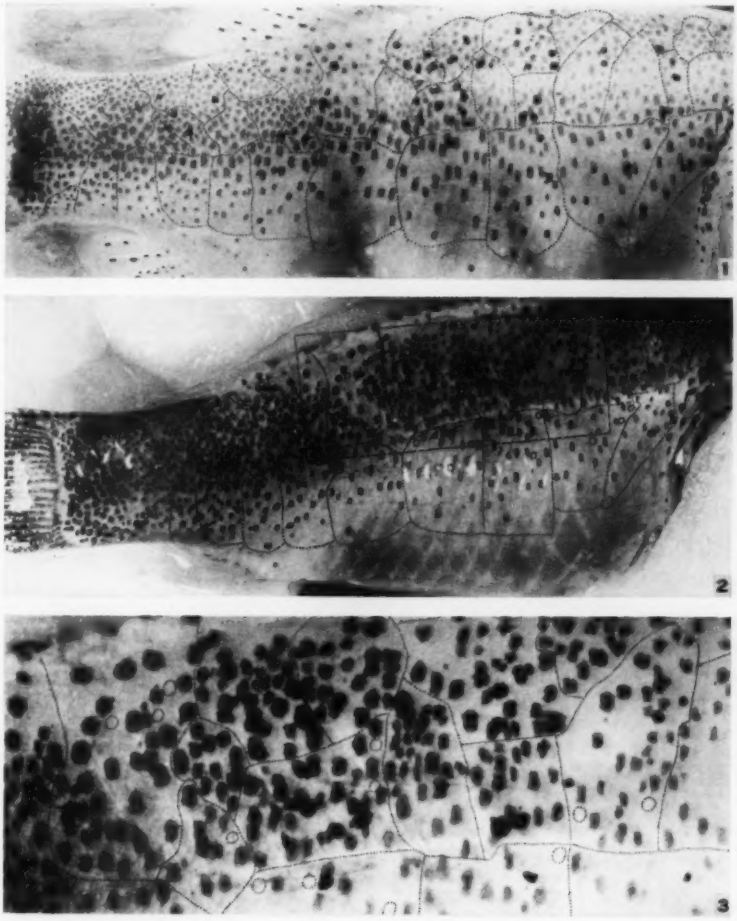

FIG. 1. Fish *MG*-3. Photograph taken August 2, 1937. × 5½. This is a "Type A" calico shubunkin; 907 cells are located in outlined areas.

FIG. 2. Fish *MG*-3. Photograph taken January 4, 1939. × 2½. Fifty-two cells have been lost and 3 new cells appeared since record of Fig. 1. Dotted circles indicate location of cells that have disappeared.

FIG. 3. Section outlined by dashes in Fig. 2 enlarged to same magnification as in Fig. 1, showing increase in size of area and of cells.

Type B

These fish show the irregular mottling which is prized by the fanciers. The dark spots are usually clusters of small melanophores too densely crowded to count. Of 97 selected on the first photograph of *MG*-4 on July 18, 1937, 46 remained on January 1, 1939 (Figs. 4 and 5). In the meanwhile, however, others have appeared and

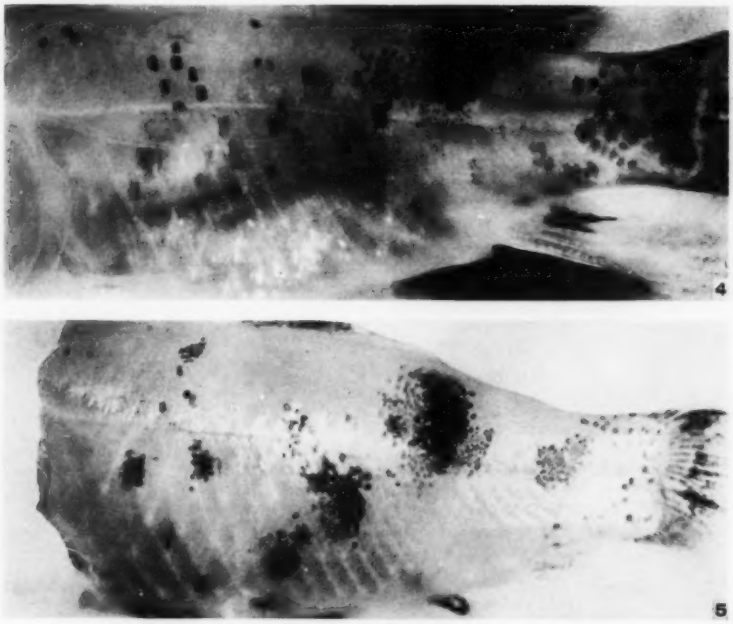

FIG. 4. Fish *MG*-4. Photograph taken July 18, 1937. × 6½.
FIG. 5. *MG*-4. Photograph taken January 4, 1939. × 2½. Fifty-one cells disappeared and 12 new cell clusters appeared in area under observation.

there has been a notable eruption of spots, or clusters of melanophores—12 altogether on the left side. These spots are first recognized as one or a few minute melanophores which rapidly increase in number. A spot for a time is often bounded by the posterior edge of a scale.

Indeterminate Types

In many cases the clusters of small cells appear among, or superficial to, cells uniformly distributed and in this way combine character-

istics of Types *A* and *B*. An example is *MG*-5, where it was possible to identify cells only in a small area. Ten disappeared out of 164 in this area, but six new clusters of cells have arisen similar to those discussed under Type *B*.

Extensive Cell Destruction

Occasionally a sweeping destruction of melanophores occurs within a few weeks. This is similar to the process in the ordinary goldfish, which is gold because melanophores but not xanthophores have been destroyed. This change most frequently takes place in ordinary goldfish at about three months of age but may occur much later (Cf. Berndt, 1925; Chen, 1925; and Goodrich and Hansen, 1931). It occurred in two of the calico shubunkins which we had under observation in this series. The history of one of these, *MG*-16, is given in Table I and in this the breakdown occurred at about eight months of age.

Increase in Size of Cells

As mentioned above, Fig. 3 shows the rectangular area marked on Fig. 2 enlarged to the same magnification as Fig. 1. The comparison of Figs. 1 and 3 then shows the actual increase in size of the area outlined. It also shows that the individual cells, which for the most part show approximately the same degree of melanin dispersion in both pictures, have definitely increased in size. It, therefore, appears that, in so far as the melanophores are concerned, the increase of body size has involved an enlargement of cells rather than a multiplication of cells.

Discussion

These observations not only show that there is much variation among individuals of these hybrids but also that each individual is variable in respect to color patterns displayed during its life cycle. The heterozygous type, as noted for earlier developmental stage by Goodrich and Hansen (1931), continues in later stages to be in a condition of unstable equilibrium between opposing tendencies—those of cell multiplication and cell destruction.

Fukui (1927 and 1930) has shown that the destruction of melanophores in the ordinary goldfish tends to take place in definitely bounded areas, giving rise to some degree of uniformity of pattern in black- and goldfish. These areas, he believes, correspond to regions of looser subcutaneous tissue bounded by more dense tissue. In effect, these may be perhaps regarded as sinuses filled with tissue fluids or lymph.

His experiments with injection of adrenalin showed a restoration of pigment which tended to be circumscribed in such areas. These results suggest that endocrine factors operating on such a region bring about under certain conditions the destruction of chromatophores and under other conditions the production of pigment. Fukui suggests that pigment destruction is due to a higher metabolic rate in these areas, but this might be stimulated by the chemical environment.

In contrast to the above, the origin of new spots or cell clusters is entirely irregular, having no relation to the areas described by Fukui. It, therefore, seems unlikely that their location can be due to endocrinal conditions.

It then seems probable that the goldfish presents a new example of the dual gene control such as has been suggested in the plumage of birds. In the case here described the direct gene action may control cell multiplication, resulting in the formation of cell clusters or spots, while remote gene control of "endocrinal regulation" may cause the destruction of cells (see Danforth, 1932, p. 33).

A discussion of the developmental origin of cell clusters will be presented in the companion paper, Goodrich and Trinkaus (p. 188).

Summary

1. The F_1 heterozygous types from the cross of the common goldfish with the transparent shubunkin (both of the species *Carassius auratus*) show not only a great range of variability between individuals, but frequently the pattern of a single individual changes markedly during the life cycle. This is due to destruction and emergence of chromatophores producing a varying pattern. It is suggested that the multiplication of cells is an example of "direct gene control" and the destruction is due to "endocrinal regulation" or remote gene control.

2. Many individual melanophores are long-lived, having been identified at the beginning and end of the 19-month period of observation.

3. Such long-lived melanophores gradually increase in size during the growth of the fish.

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THE DIFFERENTIAL EFFECT OF RADIATIONS ON MENDELIAN PHENOTYPES OF THE GOLDFISH, CARASSIUS AURATUS[1]

H. B. GOODRICH AND J. P. TRINKAUS

(From the Department of Biology, Wesleyan University)

The types of goldfish used in the following experiments are those described in the companion paper by Goodrich and Anderson (1939). These are the common goldfish, the transparent shubunkin, and the hybrid between these two known as the calico shubunkin. Genetic analysis has shown that this is a monohybrid cross and the formulae assigned have been: ordinary goldfish TT, the transparent shubunkin $T'T'$, and the calico fish TT'.

The original purpose of the ultraviolet treatment was to destroy certain parts of the color pattern in the calico fish and to study its regeneration. It was, however, soon discovered that lighter treatment than that needed to destroy the chromatophores apparently induced the formation of new pigmented areas. Consequently a more careful program of experimentation was outlined to verify these preliminary findings.

METHODS

The source of illumination has been a small laboratory mercury lamp obtained from the Hanovia Company (their model E). The quartz tube is 16 mm. in diameter, has a length of arc of 50 mm., and operates on 110-volt circuit. For purposes of destruction of melanophores, treatments frequently of 30 minutes or more were administered, but for stimulation of pigment formation most treatments were of 10 minutes duration at distances varying from 2 cm. to 6 cm. from the lamp. Only a small area was irradiated on each fish. Other parts of the body within the zone of illumination were protected. The areas treated varied from about 0.2 to 0.9 sq. cm. in size. These were delimited by pieces of wet filter paper over which were placed pieces of tin foil, which in turn were held in place by more filter paper. Wet cotton was put over the head and the operculum and over the rear of

[1] This paper is published as part of a research program at Wesleyan University supported by the Denison Foundation for Biological Research. The authors wish to acknowledge their indebtedness to Miss Priscilla Anderson who performed the preliminary experiments.

the body and caudal fin. This kept the fish moist and helped to hold it in place. The fishes were anesthetized in a 1 per cent urethane solution and were placed on a paraffin block modeled to hold the fishes nearly upright. During irradiation the spot treated was kept wet with distilled water to prevent drying of the tissue. Photographs of both sides of the fish were taken before treatment. The irradiated areas were outlined on the photographic prints and later the location of new spots was marked on these prints, or additional photographs taken if thought desirable. The fish were inspected at weekly intervals for the first three months after the treatment and those fishes that survived were observed at longer intervals for the succeeding six months.

Experiments

After the preliminary experiments, it was first planned to treat approximately equal numbers of the three Mendelian types. Accordingly, ten of each were irradiated. Later, the numbers treated were increased, especially of the hybrid type which was the only form which gave a positive reaction. The final lot of fish irradiated included 24 of the ordinary goldfish TT, 17 of the transparent shubunkin $T'T'$, and 52 of the hybrids TT', giving a total of 93 fish treated.[2] Areas with few or no melanophores were selected for irradiation. The essential result from the comparative study was that the hybrids alone showed a positive reaction by development of new melanophores, while in the two parental types no melanophores were formed. Most fish in all these groups exhibited inflammation and sometimes necrosis of tissues. In the goldfish TT the xanthophores and guanin crystals (of the reflecting tissue) were frequently destroyed. Spots or cell clusters appeared only in the hybrids. These were first observed as small faintly grayish chromatophores, having long delicate processes. The number of cells increased and in about eight weeks these cells became typical mature goldfish melanophores. (Figure 3 shows the inflammation following irradiation, and Figs. 4 and 5 the development of a cell cluster in the same spot.) Figure 1 is a photograph of a hybrid TT' taken on March 29 just before radiation and the area irradiated is outlined. Figure 2 is of the same fish on May 27. Three new spots, one small and two large, have appeared in the radiated area and one outside (in dotted circle). All but one of the new spots

[2] Eight fish of doubtful classification are excluded from these totals. Inspection of pattern indicated that they probably were one normal goldfish and seven transparent shubunkins. All gave negative reactions. Even if the presumed transparent types were transferred to the list of 52 calico shubunkins the essential results as indicated by the graphs, Figs. 6 and 7, would not be altered.

were located in the dermis superficial to the scales. This one exceptional spot was beneath the scales.

The companion paper (Goodrich and Anderson, 1939) has shown that the hybrid or calico fish is characterized by an irregular mottling and, moreover, that this pattern is subject to change during the life of the individual. It therefore seemed possible that the appearance of new spots after radiation might be nothing more than the normal sequence of events. On this account, many more of the hybrids were

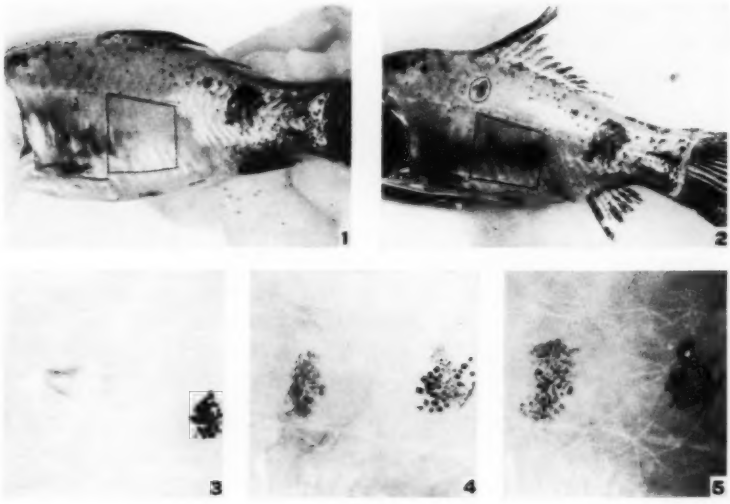

FIG. 1. Photograph of a hybrid *TT'* taken on March 29 just before radiation. The area later irradiated is outlined with dotted line. × 1¼.

FIG. 2. Photograph of same fish as in Fig. 1 taken on May 27. Three new spots (one small and two large) have appeared in the radiated area and one outside (in dotted circle). × 1¼.

FIGS. 3, 4, 5. Successive photographs of the same area on a hybrid fish. × 6. Irradiation Nov. 13, 1937. Fig. 3, appearance Nov. 27; congestion of capillaries in center (an older spot at right). Fig. 4, Jan. 2, 1938. Fig. 5, Jan. 25, 1938.

treated and the results subjected to analysis. This has shown that the irradiated areas produced a significantly greater number of spots or cell clusters than appeared on non-radiated areas. It was also found that the new spots appeared chiefly from three to six weeks after treatment with the maximum number arising during the fifth week (see charts, Figs. 6 and 7). In 24 cases two or more cell clusters appeared within the radiated area, in 17 cases only one new spot and

none were recorded in 11 cases. These results have been compared with the total number of cell clusters appearing on both sides of the body (exclusive of head and fins). The results appear significant even

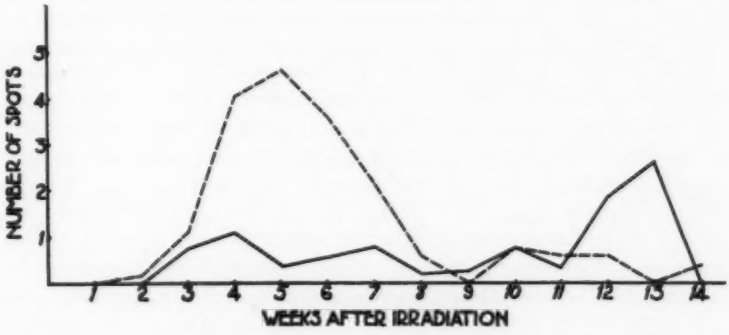

FIG. 6. Graph of numbers of spots X10 appearing in successive weeks after irradiation. Dotted line, irradiated area. Solid line, other parts of body (head and fins not included).

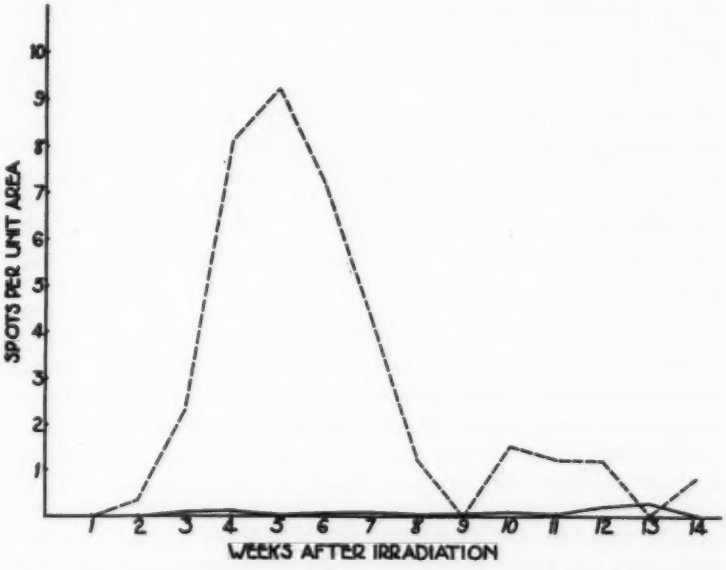

FIG. 7. Graph showing same data as Fig. 6 corrected for relative size of areas. Dotted line, numbers in irradiated area X20. Solid line, numbers on other parts of body X-1.

when no correction is made for the difference in areas compared. When, however, such a comparison is made it is found that the total non-radiated surface examined was approximately twenty times that of the average irradiated area. A graph, incorporating this correction, is shown (Fig. 7) and indicates a notable excess of development of spots in the irradiated areas. During the observational periods, from treatment until 14 weeks thereafter, there appeared a total number of 62 new spots or cell clusters within the irradiated areas and 32 outside of these areas. If we multiply by the factor 20 ($20 \times 62 = 1240$), it appears that had spots appeared at a similar rate in the non-radiated region there would have been 1240 spots, whereas there were only 32. This proportion of nearly 38 : 1 is then an index of the increased reaction of the radiated region. It is not impossible that this is an underestimate. The areas chosen for treatment were frequently below the lateral line, because this region was more clear of melanophores, and it is possible that the ventral region is one having less inherent capacity for production of melanophores.

The new cells recorded in the above experiments were in all respects similar to normal melanophores present elsewhere on the fish. Two sets of subsidiary experiments were carried out which, incidentally, gave further confirmation that these cells were normal melanophores. (1) It was found that the melanophores of the hybrid responded very irregularly to an illuminated white environment. In some cells the pigment became concentrated and in others it remained dispersed. New cells arising in the irradiated areas showed this same variability of reaction. (2) Ten scales bearing new cell clusters were transplanted to other parts of the fish as had previously been done by Goodrich and Nichols (1933) with non-radiated fish. The results were similar. The cells lived and in four cases increased, spreading over adjoining scales.

Discussion

The observations presented in this and the preceding paper (Goodrich and Anderson, 1939) show that the hybrid or calico shubunkin retains the potentiality to produce irregularly situated spots during a considerable part of the life cycle. The radiation appears to stimulate a precocious development of the spots in the areas treated. The question then arises as to what developmental or other conditions control the appearance of these spots or cell clusters. Goodrich (1927), working on the Japanese fish *Oryzias latipes*, suggested that the variegated pattern could be explained by the ameboid migration of predetermined melanoblasts of two types—that producing the maximum

amount of melanin and the other such a small amount that they remained virtually colorless. Recent investigations such as those of DuShane (1935) and Twitty (1936) on amphibia have tended to confirm the hypothesis of an early determination of wandering chromatoblasts. The paper by Willier and Rawles (1938) on the chick opens the possibility of cell determination and migration in forms where hormones have been shown to be largely operative in other phases of pigment control. The observations of Apgar (1935) on *Triturus* have suggested the concept of a widespread distribution of colorless chromatoblasts. It, therefore, seems not improbable that we may consider the calico shubunkin (especially Type *B* of the companion paper) to be invisibly spotted during development with colorless chromatoblasts—singly or in nests—and that these multiply and differentiate independently at irregular intervals to form the spots or clusters of melanophores. In some respects this hypothesis resembles the old theory of embryonic cell rests advanced to explain the cause of cancer.

In certain individual fish a wave of destruction takes place, possibly due to some hormone action, which destroys all melanophores and possibly all melanoblasts in the affected areas. We have never observed the appearance of new spots in a region which has suffered such wholesale destruction.

Attention should be called to the production in goldfish of pigment cells by X-rays (Smith, 1932). The cells appeared within a few days after treatment and disappeared a few weeks later. They did not seem to be homologous to the pattern-producing cells and resembled cells that had previously been observed arising after various mechanical injuries to the tissues (Smith, 1931). In our own experiments we have noted three cases of the formation of such cells. They were seen on the normal goldfish *TT* after unusually severe radiation from the mercury arc lamp and the appearance and history of these cells were similar to those noted by Smith.

The contrasting reactions of the three genotypes indicate that the hybrid or calico fish retains in adult condition a far greater potency to produce melanophores than either parental form. Goodrich and Hansen (1931) have pointed out that all three types form melanophores in early development. The ordinary goldfish loses these by wholesale destruction usually at about three months of age, while in the transparent shubunkin relatively few ever appear. Neither of these two parental forms produced typical melanophores when irradiated and it may be suggested that melanoblasts have also been destroyed or are

largely absent. In contrast, the heterozygous type retains the melanoblasts.

No attempt is made in this paper to determine what wave-lengths have produced the observed effect. The mercury vapor arc produces a wide range of wave-lengths. The extensive literature on effects of ultraviolet light shows that both stimulating and destructive effects have been observed. Sperti, Loofbourow, and Dwyer (1937), working on yeast cells, have suggested that cells when injured by ultraviolet liberate some growth-promoting substance, thus indicating a possible interrelation of injurious and stimulating effects. The treatments used in our experiments have been relatively more severe than those which have produced primarily stimulating effects on isolated cells. Ultraviolet light penetrates but a few millimeters through animal tissues. Sato (1933) has shown that the ultraviolet light bands characteristic of the mercury arc will pass through fish scales. The effect produced in our experiments may well be due chiefly to the regenerative processes following the inflammation and destruction of tissue.

Summary

1. Radiation from a mercury vapor lamp produced differing reactions in three Mendelian phenotypes. Two parental forms, the ordinary goldfish and the transparent shubunkin, do not develop melanophores as a result of the treatment. The F_1 hybrid, or calico shubunkin, does respond by an acceleration in the production of new spots or clusters of melanophores.

2. It is suggested that the hybrid during development becomes supplied with colorless chromatoblasts throughout the dermis which are stimulated to precocious multiplication and differentiation as a result of the radiation.

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THE REACTIONS OF THE PLANKTONIC COPEPOD, CENTROPAGES TYPICUS, TO LIGHT AND GRAVITY[1]

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INTRODUCTION

Field investigations on the vertical distribution of the plankton carried out by many different workers in recent years have established the occurrence of a diurnal vertical migration for most species of the zoöplankton. Since most investigators agree in considering light as an important controlling factor, it seemed desirable, following the work of Esterly, Spooner, Clarke and others, to attempt to study the light responses of a single planktonic species under controlled laboratory conditions.

Centropages typicus is a neritic copepod, extremely abundant off Woods Hole at certain times of the year. Clarke (1933) states that the adults show a diurnal vertical migration correlated with changes in the submarine illumination. A few preliminary observations in the laboratory showed us that the adult females are very definitely affected by light. It was therefore decided to conduct experiments on the phototropic and geotropic responses of these animals. Our choice was fortunate in that it was possible to obtain the copepods quickly and easily off Woods Hole, and to keep them at a conveniently low temperature in the laboratory to ensure their healthy existence for at least a few days.

PHOTOTROPISM: EXPERIMENTS WITH TUBES HORIZONTAL

In order to separate the phototropic from possible geotropic responses, it seemed advisable to test first the reactions of the copepods to light in a horizontal direction.

Methods

The experimental animals were obtained in Vineyard Sound by towing a scrim plankton net horizontally near the surface for about fifteen minutes. The animals, collected in the glass jar attached to

[1] Contribution No. 207 from the Woods Hole Oceanographic Institution.

the net, were poured into 3 liters of sea water and transported immediately to the laboratory. The adult female *Centropages typicus* were selected in diffuse daylight using a wide-mouthed pipette and a binocular microscope. Usually 20 healthy appearing copepods were placed in each of two glass tubes (13 × 2½″), each of which was sealed at one end with a glass plate. The open ends of the tubes were then sealed with similar glass plates, and the tubes arranged in constant temperature tanks maintained at 12° C. in the darkroom.

The two experimental tubes could be separated from each other by a distance of 21 feet. It was thus possible to obtain a wide range of light intensities for any one source, by varying the distance between the light source and the tubes. The intensities of the various inside frosted bulbs employed were as follows:[2]

Wattage	*Approximate Intensity at 1 foot*
15	13.5 foot-candles
25	25.0 "
40	43.0 "
60	75.0 "
100	150.0 "

It should be borne in mind that all the light intensities mentioned in the text are only approximate figures.

The lowest intensities used were obtained by means of neutral filters in the form of opal discs and white paper, the percentage absorptions of which were obtained by means of a photoelectric cell. Since several filters were used together at the very lowest intensities, corrections were made for diffusion and back-scattering.

Each experimental tube was marked off into quarter-lengths, and the distribution of the animals at any time, under any one condition of light, was expressed as the numbers in each section. At the very low light intensities, counting of the copepods was facilitated by lighting the tubes from behind for a moment with a weak red lamp. Preliminary tests made with this lamp showed that it had no effect on the distribution of the animals.[3]

In all the experiments, unless otherwise noted, the distribution of the animals was observed at the end of each time interval shown in the tables. After each observation, the tubes were changed, end for end, by turning them slowly in a horizontal plane. This procedure forced the animals to orientate afresh, and to redistribute themselves accord-

[2] On the advice of Mr. Eddie Kline, electrical engineer of the Canadian Laco Lamp Co., these can be considered as accurate only within 20 per cent, due to voltage fluctuation.

[3] Dr. Horton of the Department of Physics, McGill University, kindly made a spectroscopic photograph of the light emitted and found that the transmission begins at 6402 Å, and continues beyond 8600 Å.

ing to the tropistic responses actually in operation during that time interval. Enough time was allowed for the animals to establish their new distribution before a second record was taken, so that their final position was unaffected by the configuration of the previous time interval.

TABLE I

Experiment commenced at 4:00 P.M., August 19.
Tubes *A* and *B* set at distance of 5 ft. and 1 ft. respectively, from source.
At 12:00 noon, August 20, tube *A* moved to 10 ft.
At 4:00 P.M., August 20, tube *A* moved to 20 ft.
Tube *B* was kept at 1 ft. throughout.
Source: 60-watt lamp.

Time	Distance	Intensity	(Positive) * I	II	III	(Negative) IV
Aug. 19						
4:10 P.M.	5 ft.	3.0 f.c.	20	–	–	–
	1 ft.	75.0 f.c.	20	–	–	–
4:40 P.M.	5 ft.		20	–	–	–
	1 ft.		20	–	–	–
6:45 P.M.	5 ft.		16 and 4	–	–	–
	1 ft.		20	–	–	–
9:15 P.M.	5 ft.		10 and 6	–	2	3
	1 ft.		10 and 3	3	–	1
9:30 P.M.	5 ft.		8 and 6	–	2	2
	1 ft.		3 and 7	3	3	2
Aug. 20						
11:30 P.M.	5 ft.		10 and 3	1	4	3
	1 ft.		4 and 8	3	1	1
2:30 P.M.	10 ft.	0.75 f.c.	8 and 3	–	3	3
	1 ft.	75.0 f.c.	5 and 9	3	1	1
4:00 P.M.	10 ft.		8 and 4	4	4	–
	1 ft.		3 and 7	4	1	1
4:45 P.M.	20 ft.	0.19 f.c.	9 and 6	–	3	–
	1 ft.	75.0 f.c.	5 and 10	3	2	1
8:00 P.M.	20 ft.		6 and 9	–	2	2
	1 ft.		3 and 9	4	1	2

* Two numbers are sometimes given under Section I (e.g. 16 and 4). This distinguishes those copepods right at the positive end (16), from those still in this section but apparently less strongly attracted.

Observations

A series of tests (Table I) was first carried out in order to determine:

(1) The normal responses of the copepods to various light intensities within limits found in nature.
(2) The effect of continued exposure to constant light intensities over the range studied.

The results obtained (Table I) showed that the copepods were positive to all illuminations, and remained largely so after exposure.

A number of experiments was then carried out to determine the range of light intensities to which the copepods were sensitive, and to investigate the possibility of the existence of critical light intensities at which the phototropic sign might become reversed.

The copepods were found to be positive to low light intensities, the lowest to which they were attracted being *ca.* 0.005 f.c. (Table II).

TABLE II

Responses to low light intensities. Distance of experimental tube from source: 20 ft. throughout.*

Intensity	Time	(Positive) I	II	III	(Negative) IV
0.06 f.c.	Aug. 20 9:30 P.M.	5 and 4	–	2	3
" "	Aug. 21 8:30 A.M.	4 and 6	1	2	1
0.015 "	10:45 A.M.	0 and 12	–	3	1
" "	12:00 Noon	2 and 12	1	2	3
" "	3:30 P.M.	4 and 5	1	4	–
0.008 "	Aug. 24 12:40 P.M.	14	1	3	2(New animals)
" "	2:00 P.M.	1 and 10	2	2	3
" "	3:00 P.M.	3 and 9	1	3	3
0.006 "	3:30 P.M.	11	2	1	3
" "	4:50 P.M.	6	5	–	7
" "	7:00 P.M.	11	2	2	1
" "	8:00 P.M.	10	–	2	7
" "	10:50 P.M.	13	–	4	1
" "	Aug. 25 8:30 A.M.	15	1	–	–
" "	9:30 A.M.	14	–	2	1
0.005 "	Aug. 26 12:30 P.M.	9	5	4	1
" "	1:55 P.M.	10	4	3	–
" "	2:25 P.M.	3	7	6	3
0.003 "	Aug. 28 2:30 P.M.	3	4	7	6(New animals)
" "	4:45 P.M.	9	4	4	3
" "	Aug. 29 9:40 A.M.	11	6	2	–
" "	11:50 A.M.	4	6	3	6
" "	6:45 P.M.	8	3	3	3

* Each time the light intensity was changed, it was done immediately following the preceding observation.

On continued exposure to the much higher light intensities of 150 and 600 f.c. (Table III), the majority of animals on the whole exhibited a positive phototropism, although at times there were more animals in the darker half of the tube and some of the animals apparently became negative on prolonged exposure.

It seemed desirable to determine whether the copepods would be repelled by the still higher light intensity (11,380 f.c.) approximating to that of bright sunlight. As a check on the results, other copepods which had been collected at the same time were subjected to a much lower intensity of 4 f.c. The results (Table IV) show that, at least after a short exposure to this very high intensity, half of the animals became negatively phototropic, while the others remained positive.

TABLE III

Responses to high light intensities
Source: 100-watt lamp. Intensity at ½ ft. : 600 f.c.
Intensity at 1 ft. : 150 f.c.

Time	Distance	(Positive) I	II	III	(Negative) IV
Aug. 30 5:00 P.M.	½ ft.	15	–	–	3
	1 ft.	14	–	2	3
5:30 P.M.	½ ft.	13	1	1	3
	1 ft.	13	–	1	6
6:45 P.M.	½ ft.	14	1	2	1
	1 ft.	10	1	–	8
9:00 P.M.	½ ft.	14	–	1	2
	1 ft.	14	1	2	3
10:15 P.M.	½ ft.	14	2	2	–
	1 ft.	10	–	1	8
Aug. 31 9:10 A.M.	½ ft.	5	1	2	10
	1 ft.	12	3	3	2
10:20 A.M.	½ ft.	8	1	3	7
	1 ft.	10	–	2	8
11:15 A.M.	½ ft.	11	2	1	4
	1 ft.	9	–	2	8
12:15 P.M.	½ ft.	9	1	1	8
	1 ft.	10	2	3	6
1:20 P.M.	½ ft.	12	2	–	4
	1 ft.	12	2	2	4
4:00 P.M.	½ ft.	12	2	–	4
	1 ft.	8	2	2	8
5:00 P.M.	½ ft.	12	1	3	3
	1 ft.	11	2	–	7

It was rarely that all the animals displayed an invariable reaction (either positive or negative) to any one condition of light. It was possible then that some of the animals were negatively phototropic even though the majority were positive; or again, perhaps some were indifferent. To gain evidence on these points, observations were made on individuals, one being sealed within a tube. At first, observations were made for the most part once every hour, using three widely separated intensities: 3.0, 150, and 600 f.c.

At the lowest intensity (3.0 f.c.), an individual remained photopositive for four hours, but appeared to become indifferent after exposure overnight. A second individual was indifferent from the first, and remained so for 15 hours. This behaviour was not modified if the individual was left in darkness, and then exposed to the light.

TABLE IV

Source: 1000-watt lamp. Tube at 4½ inches from source.
Control tube at 20 feet.
Ice added to aquarium to offset intense heat from source.

Time	Intensity	(Positive) I	II	III	(Negative) IV
11:40 A.M.	11,380 f.c.	10	–	2	6
	4 f.c.	8	4	1	6
11:55 A.M.	11,380 f.c.	12	–	–	6
	4 f.c.	11	2	–	5
12:05 P.M.	11,380 f.c.	8	1	–	9
12:20 P.M.	11,380 f.c.	10	–	4	6
	4 f.c.	13	–	2	3
1:20 P.M.	11,380 f.c.	8	1	2	10
	4 f.c.	11	2	2	3
1:35 P.M.	11,380 f.c.	9	1	3	8
1:45 P.M.	11,380 f.c.	8	1	3	8
	4 f.c.	15	2	2	–
2:00 P.M.	11,380 f.c.	9	–	–	9
2:10 P.M.	11,380 f.c.	10	1	1	7
	4 f.c.	14	2	1	1
2:25 P.M.	11,380 f.c.	11	1	–	7
2:40 P.M.	11,380 f.c.	8	1	–	11
	4 f.c.	14	2	1	1

The responses of two individuals at an intensity of 150 f.c., and of two others at 600 f.c. were such that one individual at each intensity remained positive for 24 hours, while the other individuals were positive for the first 5 hours but apparently became indifferent after exposure overnight.

More extensive experiments on individuals were carried out, making observations every ten minutes, so long as it was possible to do so,

over a long period of time, and at a wide range of intensities (600, 150, 75, 67, 33, 13.5, 2.4, 0.87, 0.03, 0.006, and 0.002 f.c.). Of four individuals (*A*, *B*, *X*, and *Y*), specimens *B* and *Y* were strongly and constantly photopositive to all the above intensities; indeed, specimen *B* was never recorded outside Section I. Individual *A* was in the main attracted although less so at intensities above 75 f.c. Individual *X*, although less consistent, was generally attracted by the light, but occasionally at both high and low intensities it was found at the negative end of the tube, even from the beginning of the experiment.

Having studied the effects of continued exposure to different intensities, it was decided to determine the effect of changing light intensity—a condition which is more like that which occurs in nature. The changes in intensity were obtained by varying the position of the source relative to the two experimental tubes. Thus the quality of the light remained unchanged, and two experiments could be carried on at once.

Successive experiments were carried out by moving the source first 1 foot, then 2, 5, 10, and finally 20 feet every ten minutes (owing to difficulties in counting, 15-minute intervals were sometimes unavoidable). The intensities ranged from 11,380 to 4 f.c. Before the experiments were commenced, the tube at the maximum intensity was left exposed to light until a considerable percentage of the animals exhibited repulsion.

Regarding the one-foot changes: On increasing the intensity from 4 to 11,380 f.c., the animals remained continually attracted showing always at least 80 per cent in the positive half of the tube. However, after continued exposure for one hour at the highest intensity, only 40 per cent were still attracted. In the opposite tube, 55 per cent of the copepods were repelled at the beginning when the intensity was 11,380 f.c., and it was necessary to decrease the intensity to 64 f.c. before 80 per cent of the animals were attracted.

Considering the results of the 2 ft. changes, it was found that essentially similar conclusions could be reached. In the increasing intensity experiment, actually 100 per cent of the animals exhibited constant positive phototropism. Decreasing the intensity resulted in progressive attraction down to 16 f.c., when about 80 per cent of the animals were in Sections I and II. Further decreases caused little change.

The 5, 10 and 20-foot changes may be considered together. Regarding the increasing intensities, it is striking that none of the changes had any effect on altering the original distribution of the animals. The numbers of animals in each half of each tube remained almost perfectly

constant with the ten-minute intervals allowed, and it was only after prolonged exposure (45 to 60 minutes) at 11,380 f.c. that repulsion was brought about. Of the decreasing intensity experiments, in the 5-ft. changes progressive attraction resulted in 80 per cent of the animals being positive at an intensity of 7 f.c. Progressive attraction also resulted in the other experiments, with 70 per cent of the animals being attracted in the 10-ft. changes at the minimum intensity of 4 f.c. (After one hour at 4 f.c., 80 per cent were positive.)

All these experiments on different magnitudes of decrease, each occurring with 10-minute intervals, would seem to indicate that the greater the magnitude of change, the lower the intensity at which a large number of the copepods became positively phototropic. This statement may be misinterpreted unless it be remembered that undoubtedly 80 per cent, or more, of the copepods would have migrated to the positive half of the tube at much higher intensities had more time been allowed before the next change was made. (There would thus appear to be a "time-lag" effect.)

The above experiments show the effects of different magnitudes of increase and decrease with a constant time interval of 10 minutes. The percentage relationship between any one intensity and that which immediately preceded it is not by any means constant during any one succession of changes. Thus experiments were next conducted similar to the foregoing, except that there was a constant percentage increase or decrease throughout each series of changes. The actual rates of change used were such as may occur in nature. (The values chosen were the maximal changes observed by Clarke (1933) at one station in the Gulf of Maine.)

Increases and decreases of 10 per cent per hour were first tried, through a range of high intensities (11,380 to 2,840 f.c.), and then through a low intensity range (9.5 to 6.2 f.c.). Considering first the decreasing intensities, through the high range there was progressive attraction, while through the low range there was practically no alteration in the distribution. As regards the increasing intensities experiment, there was little observable change, but, if anything, a rather larger percentage of animals was attracted with time. The same result was obtained with the low intensity range. Decreases and increases of 20 per cent per hour, through both high and low intensity ranges, gave similar results.

Phototropism and Geotropism: Experiments with Tubes Vertical

Parker, Dice, Esterly, Clarke and others have demonstrated that geotropism is frequently an important factor in the vertical migration

of plankton. It seemed desirable, therefore, to carry out experiments using vertical tubes to ascertain whether the light responses would be different, and to test for the occurrence of a true geotropic reaction.

Methods

The aquaria were replaced by two large bell-jars held upright by specially constructed wooden stands. The same experimental tubes were used, but they stood vertically in all the following experiments. A lamp was suspended over each tube, and, by means of a pulley system, the distance between the lamp and the tube could be quickly altered. The maximum distance thus obtainable was 4½ feet. Whenever it was desired to illuminate the animals from below, the tubes were simply placed upright on an iron tripod, and the lamp placed underneath.

Observations

It was decided to find the effects of various rates of change of light intensity, and to compare the results with those obtained in the horizontal experiments. Unfortunately the 1,000-watt lamp burned out and as it was impossible to replace it in the short time remaining, it was necessary to confine the indoor experiments to the lower light intensities (0.67 to 240 f.c.). A wide variety of rates of change was used: 25 per cent per hour, and 25, 50, 100, 300, and 700 per cent per half-hour.

Considering the experiments on increasing light intensities the following conclusions were reached. Within the range of intensities used, it seemed that, in general, increasing the light at a variety of rates does not bring about repulsion. One experiment, however, using 25 per cent increases per half-hour, through a range from 7.4 to 19.1 f.c. did cause repulsion:—70 per cent of the copepods were attracted initially, but as the intensity increased, fewer remained positive until only 16 per cent were attracted at 19.1 f.c. A large number of other experiments, however, at intensities near 7.4 to 19.1 f.c. (also at higher and lower ranges, and at rates from 10 per cent to several hundred per cent) was carried out, and in no other case was this repulsion observed. In the great majority of cases the distribution remained almost constant. It may be then, that this single case of repulsion does not demonstrate the normal behaviour of these animals, at least under laboratory conditions.

In the experiments on decreasing light intensities, with the exception of a single experiment, decrease in intensity at all rates, and through all the ranges of intensity employed, resulted in more and more of the animals swimming to the top of the tube as the light diminished.

This progressive attraction was sometimes very great. For example, in two experiments only 25 per cent of the copepods were positive at the beginning, and nearly 90 per cent at the end. The exceptional experiment was the only one employing so low a rate of decrease as 10 per cent. It is possible that such changes are too slow to be perceptible to the animals (below threshold).

It was thought desirable to determine the effect of increasing light intensity, using direct sunlight, so that a very high intensity range would be available. The experiment was conducted in the open behind the Oceanographic Institution. An inverted bell-jar was used as in the darkroom, with the experimental tube placed inside it, standing vertically. Since it was here impossible to circulate cooled water through the bell-jar, it was simply refilled with cold sea water whenever the temperature rose. The stand holding the bell-jar was completely

TABLE V

Reactions to direct sunlight

Time	No. of Opals	Relative Sunlight	Approximate Int. in Tube (f.c.)	(Top) I	II	III	(Bottom) IV
		per cent					
12 Noon	4	100	1,080				
12:30 P.M.	4	100	1,080	11	1	4	3
1:00 P.M.	3	98	1,400	11	1	3	3
1:30 P.M.	2	93.6	2,000	12	1	1	4
2:00 P.M.	1	88	2,640	19	–	–	1
2:30 P.M.	0	79	9,470	11	2	1	5
3:00 P.M.	0	73	8,760	11	–	1	5
3:30 P.M.	4	59	636	12	–	3	3
4:00 P.M.	2	43.6	935	5	2	6	4
4:45 P.M.	0	27	3,240	7	1	3	7

covered with black tar-paper. A small aperture cut in the top allowed a beam of sunlight to fall on the top of the experimental tube. On one side of the stand, the tar-paper formed a moveable flap which could be lifted, and the necessary counts made. Four opal diffusing discs were placed over the aperture to reduce the light; these were removed at intervals. In the first experiment, they were removed one at a time, in the second two at a time, and in the last experiment all four were removed together. Each disc alone transmits 25 per cent of the light falling upon it. The beam of sunlight was directed on to the aperture above the tube by means of a simple plane mirror which could be turned as the sun changed its elevation. The light intensity was measured by means of a Weston Photronic Cell.

The results of the experiment (Table V) show that when the light had increased from about 1,000 to about 9,000 f.c. over a period of two

hours, the animals were at all times strongly photopositive. However, increases starting at lower intensities resulted in a majority of the animals in the lower half of each tube. Is there also a negative geotropism which becomes stronger with increase in light intensity? Certainly the results indicate that mere rate and direction of change of light alone cannot account completely for the movements of *Centropages typicus*.

Thus experiments were next carried out in order to test the possibility that the copepods might react to gravity, and that the above results were only partially due to phototropic responses.

The experimental tubes were placed vertically in the bell-jars in the normal way. The animals were then left in darkness, and counts made later with the red lamp. For example, the tubes were left for 1½ hours in darkness and subsequent counts gave the following results (Tubes *A* and *B* were treated identically to furnish checks on each other):

	(Top) I	II	III	(Bottom) IV
Tube *A*	14	4	1	–
Tube *B*	6	4	–	8

The tubes were then reversed vertically end for end. After one-half hour the following results were obtained:

	(Top) I	II	III	(Bottom) IV
Tube *A*	12	5	3	–
Tube *B*	12	3	–	3

The tubes were again reversed. After one-half hour the following results were obtained:

	(Top) I	II	III	(Bottom) IV
Tube *A*	11	3	1	2
Tube *B*	15	1	–	3

The above results clearly show that the animals are on the whole negatively geotropic in darkness. Careful observation showed that the animals sink rapidly if they cease swimming. Hence actual effort was necessary for them to remain at the tops of the tubes, and the geotropism must then be quite strong.

The relation between geotropism and phototropism was then tested by taking the above animals from darkness and illuminating them from below, with the following results:

	(Top) I	II	III	(Bottom) IV
Tube *A* (15-watt lamp below)	7	4	3	4
Tube *B* (100-watt lamp below)	–	1	–	19

The results would indicate that negative geotropism is stronger than positive phototropism when the light is weak, while positive phototropism is overwhelmingly strong when the intensity is high.

Tube *B* was returned to darkness and a count 15 minutes later showed that the majority of animals were in Section I.

These results verified the negative geotropism. A 60-watt lamp was then placed below the tubes and the following results obtained:

	(Top) I	II	III	(Bottom) IV
Tube *B* (60-watt lamp below the tube).....	1	–	–	19

Both tubes were again returned to darkness and a count 45 minutes later again showed a large majority exhibiting negative geotropism. A 25-watt lamp was then placed below the tubes:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below the tube).....	4	1	3	9

The experiment was repeated. The animals again showed negative geotropism in darkness. With a 25-watt lamp below the tubes the results were as follows:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below the tube).....	4	1	–	12

Finally it was decided to determine the effect of replacing a low light intensity by a high one, when the geotropism and phototropism were in opposition. It has been shown that after exhibiting negative geotropism in darkness, on exposure to a 25-watt bulb from below the distribution was:

	(Top) I	II	III	(Bottom) IV
Tube *B* (25-watt lamp below).....	4	1	–	12

This lamp was then replaced by a 100-watt lamp. A count after 15 minutes showed:

	(Top) I	II	III	(Bottom) IV
Tube *B* (100-watt lamp below).....	–	–	–	17

All the above experiments definitely establish that the adult female *Centropages* is primarily negatively geotropic and positively phototropic. When the two are acting in opposition, the positive phototropism becomes progressively stronger as the light intensity increases.

Discussion

It is still a controversial matter how far laboratory experiments of the type conducted are applicable to conditions in nature. Throughout all the experiments, however, it was our aim to avoid "shock"

conditions, and the use of surface tow-nettings avoided large changes in light intensity during the collections.

It would seem from the experiments with artificial light, that adult female *Centropages typicus* should be right at the surface during most of the day, since they are strongly positively phototropic to a very wide range of light intensities, and it does not seem that continual decreases are always necessary to cause a majority to remain positive, such as was found to be the case with *Acartia clausi* (Johnson, 1938). However, repulsion does occur to some extent on prolonged exposure to very high intensities, and also in the experiments using direct sunlight (Table V) when the illumination increased through such ranges of low intensities as may occur in the early morning. Hence, after considerable exposure to strong sunlight (about midday in summer) and possibly also when the light is increasing in the early morning, *Centropages* might be expected to be a little lower in the water.

G. L. Clarke (1933) however, found that these copepods have a maximum of about 13 m. during most of the day in the Gulf of Maine. Some hauls made in August, 1935, near Woods Hole, were examined and these in general confirmed this finding, although there were cases when the majority were at the surface. (Clarke also did find, for two stations, the majority at the surface.)

In considering this difference it must be remembered that there are other factors acting in nature. Thus, especially at the surface, turbulence may carry the copepods to somewhat lower depths. Further, the possibility of muscular fatigue must not be overlooked. As has been mentioned, *Centropages* will sink rapidly as soon as it ceases swimming, and thus some will tend to sink below the surface, though positively phototropic. This probably accounts for the observation, that, although using the same intensities, a considerably larger percentage of animals is found in the negative half of the tube in the vertical experiments than in the horizontal ones. It should also be noticed that Clarke did find a secondary maximum of *Centropages* at the surface.

The rise to the surface at night, observed by Clarke and others, is explainable since *Centropages* is always very strongly attracted when the light intensity is diminished. The negative geotropism, evident at least during and just after exposure to darkness, will aid the rise.

Parker (1901) found that female *Labidocera* migrate surfacewards at night due to positive phototropism and negative geotropism, and Dice (1914) considered geotropism the major factor in the migration of *Daphnia*. However, the recent findings of Kikuchi (1938) exemplify

the fact that the actual rôle played by geotropism probably varies from species to species.

Since *Centropages* is positively phototropic to very low intensities, the upward migration will presumably continue when the light is exceedingly weak. Further, when the animals have reached the surface, they will tend to remain there during darkness owing to the negative geotropism, and they will not take up a more or less uniform distribution, as Russell has supposed for some planktonic species.

As regards the downward migration in the morning, we were generally unable to demonstrate repulsion with increase in light intensity using electric light in the laboratory. However, in the experiments using direct sunlight, it was shown that increase in intensity at a low intensity range from about 700 to 3,500 f.c., did cause repulsion, and this range of light change might be expected in the early morning. It is possible that exposure to darkness during the night might also tend to render the animals more sensitive to light, but there is the opposing geotropism to consider. This has been shown, however, to be definitely weaker for average light intensities. Further experiments, however, are desirable in this connection.

Although no experiments were conducted to test specifically Esterly's theory of a diurnal rhythm (Esterly, 1917, 1919), it would seem from an examination of our readings at different times of the day that such a rhythm is absent in *Centropages*. Rose considers that a species exhibiting diurnal vertical migration is adapted to a certain optimum light intensity (Rose, 1925). Many investigators have been unable to demonstrate such optima in the laboratory. Esterly, for example, found *Calanus* negative to all intensities used, provided the temperature was above 10° C. Rose believed that if a wide range of intensities was employed in the experiments, the optima would be demonstrable. We therefore used a very wide range in our experiments, but did not find any such optimum for *Centropages*.

Reversal of phototropic signs with absolute intensity of light was also difficult to obtain, though Loeb, Parker, Rose, etc. have demonstrated this for many planktonic species. It should be noted that Clarke also found there was no evidence from his experiments for an optimum light intensity in *Daphnia*. He also found that reversal of phototropic sign could not be brought about by absolute light intensity in this form. (Clarke, 1930 and 1932.)

Various authors have frequently pointed out the complexity of the problem of vertical migration by showing differences in behaviour between different species (e.g. Clarke, 1933), between the sexes of a single species (e.g. Russell, 1928), and between ages of the same sex

of one species (e.g. Gardiner, 1933). The observations of the authors of the present paper further illustrate that although the majority of adult female *Centropages typicus* do behave in a similar manner, variation in vertical distribution between individuals may be expected even when they are of the same species, sex and age. This is in agreement with field studies.

Summary

Experiments on phototropism and geotropism in adult female *Centropages typicus* were conducted. The following conclusions were indicated:

A. Experiments with experimental tube horizontal.

1. The copepods are primarily photopositive and constant exposure does not modify this reaction except at very high intensities in the neighborhood of that of bright sunlight (*ca.* 12,000 foot-candles) when a large number exhibited negative phototropism after continual exposure for about an hour.

2. The lowest intensity at which there were always more copepods in the brighter than the darker half of the tube was *ca.* 0.005 f.c.

3. There are two types of individuals. One type, after continuous exposure to light, becomes indifferent. In the other type, the animals are persistently attracted.

4. Decrease in light intensity, at a variety of rates and at a wide range of intensities, always results in increased attraction.

5. Increase in light intensity, at a variety of rates and at a wide range of intensities, has no effect on the behaviour. Only prolonged exposure at high intensities repels the animals.

B. Experiments with experimental tube vertical.

1. With the light from above the animals stay mainly at the top of the tube through a wide range of intensities, a distribution which is probably the result of positive phototropism, negative geotropism, or both.

2. Increases in intensity have no effect on the animals except when sunlight is used. A fair percentage of the animals is then repelled.

3. With the exception of decreases as low as 10 per cent per hour, decreases in intensity result in increased attraction.

4. The animals are strongly negatively geotropic in darkness. When geotropism and phototropism are opposed, the reactions depend upon the intensity of the light.

5. The possible bearing of these conclusions on the vertical distribution and diurnal vertical migration of adult female *Centropages typicus* is discussed.

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EMBRYONIC INDUCTION IN THE ASCIDIA

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INTRODUCTION

The Ascidia are grouped with those animals whose early development is termed mosaic. Yet, in the closely related Vertebrata, organs form as the result of interaction between those cells which become the definitive organ in question and neighboring cells, whose descendants take no part in the actual formation of the organ. The independent differentiation of organs in the Ascidia and the dependent differentiation of the same organs in the Vertebrata presents a problem.

It is believed that the Ascidia and the Vertebrata are descendants of a common ancestor which contained organs similar to those now common to both groups. The nerve cord, for example, in the two groups is thought to be homologous. The common ancestor must have had a nerve cord which arose either under the influence of inductors or independently as a mosaic piece. It seems strange that in the course of evolution the vertebrate nerve cord and the ascidian nerve cord could have remained such similar embryonic structures, when their modes of origin were diverging so greatly that the one now forms under the influence of inductors and the other quite independently of inductors. Possibly this difference in mode of development between the two groups is more apparent than real.

The injury experiments of Conklin (1905*b*) established the fact that in *Styela partita* surviving blastomeres do not deviate from their prospective potency by regulating to form more morphological units than they normally form as parts of a whole embryo. Conklin's work further showed that differences in protoplasmic appearance and cleavage peculiarities develop in the uninjured blastomeres just as they would were the blastomeres part of an intact embryo. These differences are numerous enough to allow a careful observer to differentiate the presumptive tissues and organs before the formation of the definitive structures. This fact, I think, is the basis for the belief that the ascidian egg is a mosaic of self-differentiating parts. The isolated parts certainly self-differentiate into what are recognizably distinct presumptive regions, but the question is whether any or all of these isolated presumptive regions are capable of further self-differentiation into embryonic organs.

The problem, then, is to determine whether or not inductive influences are present in the developing ascidian embryo, and, if they are present, which cells release inductors and which structures develop in dependent fashion. The answers have been sought with the aid of isolation and transplantation techniques.

I wish to express deep gratitude to Dr. Barth for his valuable suggestions during the course of this work.

Materials and Methods

The animal chosen for these experiments was *Styela partita*. This particular animal was used because its normal embryonic development is comprehensively portrayed and because the mapping of presumptive regions is complete (Conklin, 1905*a*). A further reason for using the egg of *Styela* is that much of the experimental work on early ascidian development with which the present work must be compared was done on this egg.

Fertilized eggs were obtained in two ways. In the early part of this work several animals were cut in two and the eggs and sperm removed from the gonads and ducts in a pipette and mixed in sea water. Only a small percentage of eggs was fertilized. These were recognized by the cap of concentrated yellow pigment which forms after fertilization and were sorted out for use. This method is laborious and leaves little time before the first cleavage occurs in which to prepare the eggs for operations. Fertilized eggs are obtained more easily and quickly from spawning animals. Usually *Styela* spawn in the laboratory some time between 4 and 7 P.M. However, they may be induced to spawn at any time of day or night by subjecting them to light for eleven or twelve hours preceding the desired time of spawning. The animals were kept in running sea water aquaria where the light was controlled with an opaque oil-cloth cover and an electric light. Bulbs of 40 and 150 watts placed directly over the tank and about eighteen inches from the animals were found to be equally effective. As a rule the aquarium was shaded with the oil-cloth during the afternoon and evening and the light turned on at about 10 P.M. The animals then started to spawn the next morning between nine and ten. The time between the spawning of the first animal and the last varied from fifteen minutes to several hours. During the longer spawning periods eggs were collected for use several times. The same group of animals could be induced to shed clouds of sperm and eggs on four or five successive days, by controlling the illumination.

There is something released into the water by the spawning animals

which induces others to spawn, provided that the latter have had almost the necessary eleven or twelve hours of light. This knowledge was used occasionally in causing the animals of one tank to spawn several hours before the expected time by adding some water from another tank in which spawning had ceased shortly before.

Eggs were carried in small pipettes through eight washes of pasteurized sea water. The water had been heated to 70° C. and maintained at that temperature for five or ten minutes. After cooling it was aerated by shaking and used immediately or kept in the refrigerator overnight until shortly before use the next day. The operating dishes were 20 mm. Stender dishes. These were flamed with a Bunsen burner each time before use and a hot 1.5 per cent agar solution in sea water was permitted to cool and solidify upon the bottom of each dish. The smooth agar surface prevented the eggs from adhering to the glass. The operating solution was 0.4 per cent 0.1N HCl in pasteurized sea water, which changes the pH to approximately 7.6. The sea water was slightly acidified because most of the eggs, after removal from the membranes in ordinary sea water, cleaved abnormally and often cleavage furrows disappeared although nuclear divisions continued, very much as is the case when *Arbacia* eggs are treated with alkaline or acid sea water (Smith and Clowes, 1924). Acid rather than base was tried because Child (1927) had found more normal development of *Corella willmeriana* embryos outside of the atrium when the CO_2 tension was increased. Child found the pH of the atrium to be approximately 7.4. In the acidified sea water injury from manipulation was much less frequent. The eggs seemed more viable and, without membranes, could develop into normal tadpoles not distinguishable from those grown within the protective membranes. Pasteurized sea water and semi-sterile precautions with operating dishes and instruments were employed because survival with good differentiation was increased from about 10 per cent to over 90 per cent by so doing. Instruments were dipped in alcohol between operations and the pipette shaft flamed each time after use.

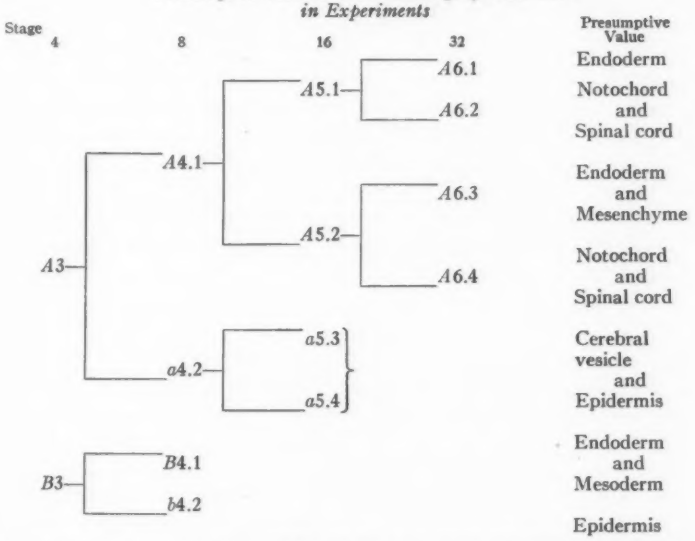
Membranes were removed from the eggs in operating dishes with the aid of fine watchmakers' forceps. The denuded eggs were then transferred to other operating dishes in finely tipped pipettes. Blastomeres were separated with Spemann glass needles. Transplantations were accomplished by simply bringing one cell or group of cells to rest upon another group with which combination was desired. The cells of the cleavage stages are quite sticky and adhere readily.

Embryos were fixed in Bouin's fluid. After fixation they were transferred to a 1.2 per cent agar solution as it was cooling. After solidification of the agar, blocks containing the embryos were cut out

and passed through the alcohols. The 95 per cent alcohol through which the blocks were passed during dehydration contained some water-soluble eosin. The blocks and the embryos were stained enough so that they might be seen more easily during clearing and imbedding. This is a modification of a technique employed by Dalcq (1932) for the manipulation of ascidian embryos. Sections of seven micra were cut and then stained a few minutes in Heidenhain's haematoxylin at 45° C. after a previous mordanting of twenty minutes in 4 per cent ferric alum. Further staining for three minutes in 1 per cent light green after treatment in 0.5 per cent phosphotungstic acid for five minutes was sufficient to stain the yolk material. A 0.5 per cent solu-

TABLE I

Presumptive Value and Cell Lineage of Cells Used in Experiments



tion of eosin in slightly acidified 95 per cent alcohol counterstained sufficiently in thirty seconds.

The photomicrographs were taken through an oil immersion lens. I wish to thank Mr. J. Godrich for his part in the preparation of the photographs and plates.

EXPERIMENTAL SECTION

In Table I the presumptive value and cell lineage of the cells used in the experiments to be described are given. The presumptive value and cell lineage were worked out by Conklin (1905*a*). The relative positions of the cells described in Table I may be seen in Figs. 1–4.

In both the table and figures the cell notations are given for only one side of the embryo, since the cleavage pattern is bilaterally symmetrical. When reference is made to corresponding cells of both sides of the embryo, the figure 2 is placed before the cell lineage notation. A figure greater than 2 indicates that corresponding cells of more than one embryo have been used.

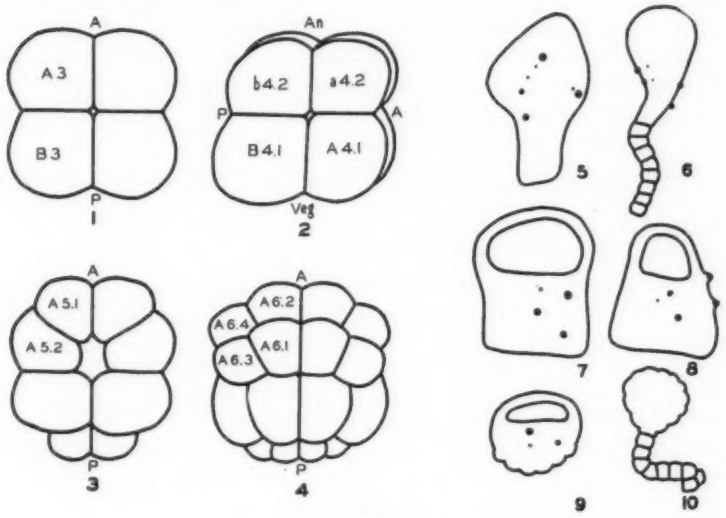


PLATE I

Abbreviations: *A*, anterior; *An* animal pole; *P*, posterior; *Veg*, vegetal pole. The cell lineage notations may be understood by referring to Table I.

FIG. 1. A vegetal view of a four-cell stage.

FIG. 2. A right side view of an eight-cell stage.

FIG. 3. A vegetal view of the eight vegetal cells of a sixteen-cell stage.

FIG. 4. A vegetal view of the sixteen vegetal cells of a thirty-two cell stage.

FIGS. 5–8. Surface views of anterior half, 2*A*3, embryos. Supernumerary pigment spots are present in all and a bare notochord is shown in Fig. 6.

FIG. 9. A surface view of an anterior quarter embryo, 1*A*3.

FIG. 10. A surface view of an anterior vegetal quarter embryo, 2*A*4.1. A notochord is present.

The early cleavages allow an experimental isolation of the presumptive regions and combinations of various regions in order that the normal interactions may be ascertained.

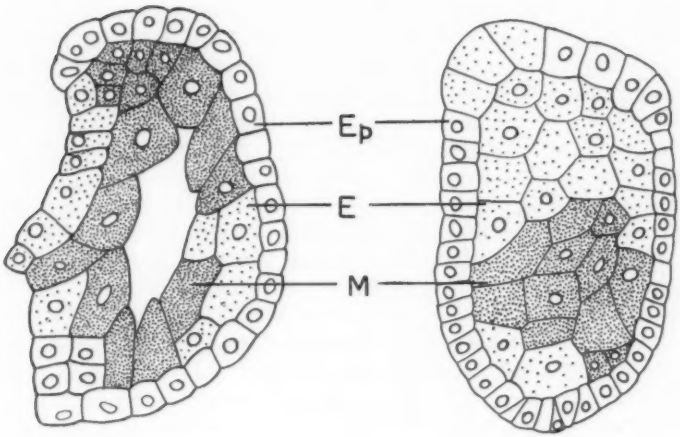
Comparison of Anterior and Posterior Half Embryos

Separation of the yellow, *B*3, and gray cells, *A*3, in the four-cell stage serves to test to what extent the two may differentiate inde-

pendently of each other. Both parts have been shown, by Chabry (1887) in *Ascidiella*, and by Conklin (1905*b*) in *Styela*, to undergo partial cleavage and to gastrulate. The anterior or gray cells may form a notochord, neural structures including the pigmented sensory cells (otolith and eye-spot), and endoderm which in some cases becomes arranged in the form of a gut with lumen. Figure 13 is a drawing of a section of an anterior half embryo showing the above-mentioned features. The high degree of differentiation of the anterior partial embryos is in agreement with the results of Chabry and Conklin.

A peculiarity shown by approximately half of these anterior half embryos, grown from 2*A*3 cells, is the presence of more than the normal two sensory spots. Figures 5–8 show surface sketches of four such embryos. Figure 9 shows an anterior quarter embryo grown from 1*A*3. The greatest number of pigment spots observed in the anterior half embryos was nine. Often pigment formed in cells widely separated, sometimes on opposite sides of the embryo. In many of the anterior embryos the neural plate did not fold over to form a cerebral vesicle, but, instead, remained on the surface of the embryo. This was usually the case when supernumerary sensory spots were formed. Figures 15 and 16 are adjoining sections of a 2*A*3 embryo which has an infolded embryonic nervous system. One of the pigment spots is external and three are internal. One of the internal sensory cells was cut in such a way as to be included in both sections. It is readily seen that more pigment is produced by anterior half embryos than would be produced by such cells when part of a whole embryo. The presence of extra sensory cells has also been observed in unoperated embryos. Here, however, their occurrence is rare, and never more than four have been seen in one embryo. The phenomenon of supernumerary pigment spots will be further discussed below.

Contrasted with the rather complete differentiation of the anterior half embryo is the unorganized condition of the posterior half. Gastrulation occurs and the embryos survive past the time when the controls become swimming tadpoles, but the presumptive muscle cells remain large and almost round, never elongating nor taking on the fibrous appearance of muscle cells. Figure 11 is a drawing of a section of a posterior half embryo showing the absence of differentiation. Chabry (1887) cultured posterior half embryos of *Ascidiella* and found poorer development of posterior than of anterior halves. No mention was made of muscles. Conklin (1905*a*, p. 52, footnote), employing the convention of calling a cell a "muscle cell" if, in normal development, it would give rise to nothing but muscle, designated these undifferentiated cells of the partial embryos, muscle cells. Since this work is



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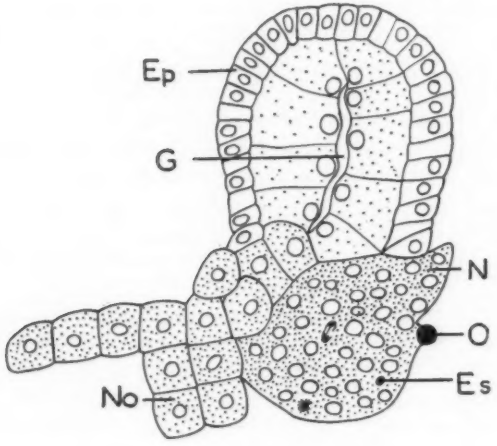

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PLATE II

Abbreviations: *Es*, eye-spot; *Ep*, epidermis; *E*, endoderm; *G*, gut; *M*, mesoderm; *N*, neural tissue; *No*, notochord; *O*, otolith.

FIG. 11. A drawing of a section of a posterior half embryo, 2*B*3, containing epidermis, mesoderm and endoderm.

FIG. 12. A drawing of a section of a posterior vegetal plus anterior animal embryo, 2*B*4.1 + 2*a*4.2, containing epidermis, mesoderm and endoderm.

FIG. 13. A drawing of a section of an anterior half embryo, 2*A*3, containing epidermis, gut, notochord and neural tissue with otolith and eye-spot.

concerned with the problem of differentiation, such cells are considered as presumptive, and the term "muscle cell" is reserved for those cells which attain the stage of differentiation found in contractile tissue and acquire myofibrillae.

The isolation and study of twenty-seven anterior and posterior embryos have shown that the gray cells, the *A3*, contain within themselves the ability to self-differentiate, whereas the yellow cells, the *B3*, lack something which would enable them to differentiate.

Animal and Vegetal Embryos

It is possible to observe the development of presumptive epidermal and cerebral vesicle cells isolated from mesodermal and endodermal cells by separating the animal from the vegetal blastomeres in the eight-cell stage. In forty-five such cases there was never evidence of neural differentiation in either the animal or vegetal half. Nothing like a neural tube formed, nor did sensory cells develop. The picture one obtains from sections of partial embryos of the animal region is one of undifferentiated cells showing no cerebral vesicle (Figs. 17–19). Instead of a row of epidermal cells surrounding a vesicle of neural tissue bearing pigment spots in two of the cells, the isolated animal embryos show nothing but a group of closely packed similar cells usually arranged about a cavity. This cavity formed between the dividing cells before the time of formation of neural tissue at the time when control embryos were gastrulae. Some of the animal embryos have a wrinkled appearance and instead of a single cavity, contain several.

Tung (1934), performing the same operation in *Ascidiella scabra*, obtained animal embryos, some of which he believed contained neural tissue. These embryos showed folds or depressions, the cells of which stained more heavily with eosin than did the other cells, or contained a few cells grouped together making a small tube. Since the cerebral vesicle in normal embryos stains more readily with eosin than do the other tissues, and since neural structures arise through a folding process, Tung thought his animal embryos possessed neural tissue. The stain criterion may be reasonably doubted. Tung shows that the presumptive neural cells in the gastrula stage are already eosinophil. At this time the cells are undifferentiated. It seems inadvisable, therefore, to use the eosinophil nature of the cells as a criterion of neural differentiation.

Conklin (1905*b*) also recognized neural tissue in isolated anterior animal cells, but used different criteria. His criteria were that the cells in question in the living condition were very clear cells, as are the

neural plate cells of a whole embryo, and, further, that their cell lineage and size were the same as the neural plate cells of a whole embryo. These criteria of neural differentiation seem valid for only the very beginning of differentiation of neural cells. A better criterion would be the formation of a structure more like the normal cerebral vesicle, a vesicle bearing an otolith or an eye-spot. Such has never been recorded from isolated animal cells. Never in past work (Chabry, Conklin, Tung), nor in the present work have sensory structures been seen to develop in isolated animal cells. It seems, then, that there must be some factor extrinsic to the presumptive brain cells which enables them to differentiate.

Isolation of the vegetal quartet of blastomeres, 2*A*4.1 + 2*B*4.1, in the eight-cell stage should test whether vegetal cells are able to self-differentiate. Few vegetal half embryos survived until the time when differentiated structures might be expected. The great majority continued to cleave until gastrulation time. Then the embryos became loosely adhering masses of cells which soon disintegrated. One, however, remained intact long enough to produce a differentiated notochord. The vegetal embryos of *Ascidiella* produced by Tung (1934) show a higher degree of differentiation.

Notochordal cells have also been seen to form in quarter embryos derived from the anterior vegetal cells alone, the 2*A*4.1. Figure 9 is a surface view of such an embryo showing the bare notochord.

Abbreviations: *A*, cup of animal cells; *M*, myofibrillae; *No*, notochord; *Ot*, otolith; *V*, plug of vegetal cells.

FIG. 14. A section through the cerebral vesicle of an unoperated tadpole, showing the size of the larger pigment spot, the otolith.

FIG. 15. A section through the pigment spot region of an anterior half embryo, 2*A*3.

FIG. 16. An adjoining section of the same embryo shown in Fig. 15.

FIG. 17. A section through an animal half embryo, 2*a*4.2 + 2*b*4.2, showing several cavities.

FIG. 18. A section through an animal half embryo, showing the unorganized nature of the embryo.

FIG. 19. A section through an anterior animal quarter embryo, 2*a*4.2, showing epidermal vesicle formation.

FIG. 20. A section of a 2*A*4.1 + 2*b*4.2 embryo through the induced cerebral vesicle and otolith.

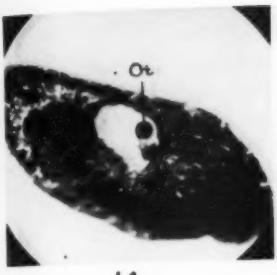
FIG. 21. A section through a 2*A*4.1 + 2*b*4.2 embryo, showing the induced pigment spot.

FIG. 22. A section through a 2*a*4.2 + 1*A*5.2 embryo. The otolith is attached to the cup of cells which arose from the 2*a*4.2. Inserted in the concavity of the cup may be seen the plug of cells derived from the *A*5.2 cell.

FIG. 23. The section passes through a cerebral vesicle containing a typical otolith in a 2*a*4.2 + 2*A*5.1 combination.

FIG. 24. A tail section of an unoperated embryo. The central notochord is flanked by the rows of dark myofibrillae.

FIG. 25. A tail section of a posterior three-quarter embryo, showing the notochord flanked by rows of dark myofibrillae.



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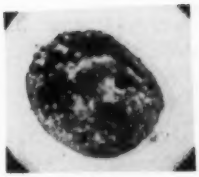

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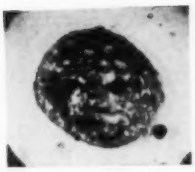

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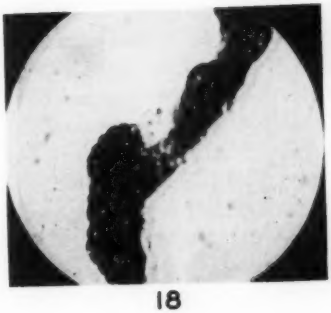

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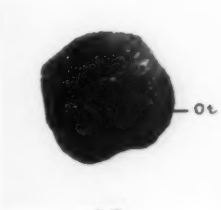

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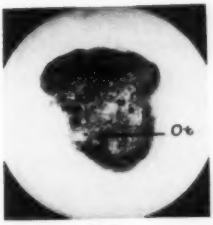

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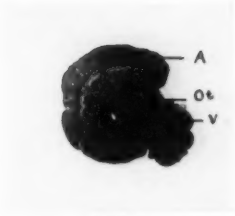

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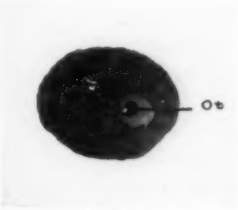

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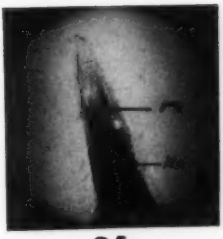

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PLATE III

The isolation experiments seem to indicate that factors or inductors necessary for the differentiation of other parts of the embryo are located in the anterior vegetal region. The evidence for this is the absence of differentiation in embryos which lack this region and the higher degree of differentiation of embryos which contain the anterior vegetal material.

Transplantations

A more striking and positive demonstration of an inductor is obtained when the inductor region in combination with cells incapable of self-differentiation causes those cells to form a structure which neither the inducing nor the reacting cells would form in normal development. Combinations of $2A4.1 + 2b4.2$ from the eight-cell stage have led to the development of partial embryos possessing cerebral vesicles and sensory cells. Figures 20 and 21 are sections of two such embryos through the otolith region. The number of $A4.1 + b4.2$ combinations which produced embryos containing pigmented sensory cells was fifteen out of forty-six. In these embryos presumptive epidermis cells have replaced presumptive cerebral vesicle cells, and, in combination with inductor, have formed cerebral vesicles and the pigmented sensory cells.

An attempt to determine the extent of the cerebral vesicle inductor in the anterior vegetal quadrant has been made. The $A5.1$ and the $A5.2$ cells of the sixteen-cell stage and the $A6.1$ and $A6.3$ and the $A6.2$ and $A6.4$ cells of the thirty-two cell stage have been combined with animal cells of the eight-cell stage. Both the $A5.1$ cells and the $A5.2$ cells have induced cerebral vesicles and sensory cells. Figure 23 shows a well-formed otolith in a cerebral vesicle. This embryo arose from a $2a4.2 + 2A5.1$ combination. Figure 22 is a section through a $2a4.2 + 1A5.2$ embryo. It is of interest because it shows that the otolith formed from an animal cell which was in direct contact with $A5.2$ derivatives. The animal cells are seen in the form of a cup with a plug of vegetal cells protruding from the concavity of the cup. A few combinations were made in which one member of the pair was stained with Nile Blue Sulphate. In five instances gastrulation was incomplete and in these the sensory cells formed on the surface at the boundary between the stained and unstained portions. The proximity of the inducing vegetal cells and the reacting animal cells suggests a direct transfer of inducing substance from the vegetal to the animal cells.

The extent of the inductor in the thirty-two cell stage is less clear. One thirty-two cell embryo from which the $2A6.2 + 2A6.4$ cells were

removed produced a sensory pigment cell. This was the only operation of this type performed. In this embryo the only anterior vegetal cells present were derivatives of the 2*A*6.1 and 2*A*6.3 cells, presumptive for endoderm and mesenchyme. The presumptive notochord and spinal cord cells were removed. A few other operations testing for the presence of neural inductor in the *A*6.1 and *A*6.3 derivatives were performed late in the operating season during September when sensory pigment was not forming in the operated embryos, or even in a number of the control embryos. The combination was *A*6.1 + *A*6.3 + 2*a*4.2. Of four successful combinations, two showed evidence of neural invagination. One of these two contained a solid internal rod of neural type cells. Of four embryos resulting from *A*6.2 + *A*6.4 + 2*a*4.2 combinations, none showed any evidence of neural invagination. The negative cases are so few here and the criteria of neural differentiation so limited that our knowledge of the neural inducing ability of the *A*6.2 and *A*6.4 cells remains uncertain. The *A*6.1 and *A*6.3 cells in the whole embryo give rise to the endodermal cells which directly underlie the cerebral vesicle. It is probably they, in normal development, which induce the cerebral vesicle.

Twenty combinations of 2*B*4.1 + 2*a*4.2 gave no evidence of neural differentiation (Fig. 12). The embryos are very similar in appearance to posterior half embryos, 2*B*3 (Fig. 11). This result indicates that the neural inductor is limited to the anterior vegetal region and does not spread over the entire vegetal region.

The extrinsic factors functioning in muscle differentiation will be described in a future paper. At present, it may be said that the presumptive muscle cells, when they are part of a posterior half embryo, do not self-differentiate (Fig. 11). Neither do they differentiate when combined with anterior animal material, 2*B*4.1 + 2*a*4.2 (Fig. 12). Functional tail muscles do form, however, in posterior three-quarter embryos. The operations were performed in the thirty-two cell stage when the 2*A*5.1 + 2*a*5.3 cells were removed, leaving the 2*a*5.4 and 2*A*5.2 cells in combination with the posterior half of the embryo. Figure 11 is a photograph of a section of an unoperated embryo's tail, and Fig. 12 is a similar section of a tail of a posterior three-quarter embryo. Myofibrillae may be seen in both sections.

Potency to Respond to Cerebral Inductor

The relative potency to respond to the cerebral inductor has been found to differ in various parts of the embryo. Table II is a summary of the data. The normal number of sensory pigmented cells found in the cerebral vesicle of *Styela* is two. Rarely four appear. Blasto-

mere combinations which are predominantly of anterior materials regularly produce sensory cells. These cells, which form in normal development in the brain, may be considered to be evidence of the presence of neural differentiation, even though in many cases a neural tube has not formed. The number of sensory cells which developed

TABLE II

Reaction to Cerebral Inductor

Combination	Presumptive Value	No. with Sensory Cells	No. without Sensory Cells	No. of Sensory Cells
2*A*3	*Ep, CV, Not, SC, G, Mes.*	22	2	1–9
2*a*4.2+1*A*5.1	*Ep, CV, Not, SC, G.*	2	0	1–4
2*a*4.2+2*A*5.1		4	0	1–4
2*a*4.2+2*A*5.2	*Ep, CV, Not, SC, G, Mes.*	5	1	1–4
2*a*4.2+1*A*5.2		1	0	1
2*a*4.2+2*b*4.2+2*A*5.1	*Ep, CV, Not, SC, G.*	2	3	1–2
2*a*4.2+2*b*4.2+1*A*5.1		1	0	2
2*a*4.2+2*b*4.2+2*A*5.2	*Ep, CV, Not, SC, G, Mes.*	3	1	1–4
2*a*4.2+2*b*4.2+1*A*5.2		1	2	2
2*A*3+1*B*4.1	*Ep, CV, Not, SC, G, Mes, Mus.*	3	2	2–3
1*b*4.2+2*A*4.1	*Ep, Not, SC, G, Mes.*	3	0	1–2
2*b*4.2+2*A*4.1		7	35	1–2
2*b*4.2+1*A*4.1		3	6	1–2
4*b*4.2+2*A*4.1		1	0	2
5*b*4.2+2*A*4.1		1	0	1
2*b*4.2+1*A*5.1	*Ep, Not, SC, G.*	0	1	–
2*b*4.2+2*A*5.1		0	3	–
2*b*4.2+1*A*5.2	*Ep, Not, SC, G, Mes.*	0	1	–
2*b*4.2+2*A*5.2		0	3	–
1*b*4.2+2*A*5.2		1	0	1
2*B*4.1+2*b*4.2+1*A*4.1	*Ep, Not, SC, G, Mes, Mus.*	0	8	–

CV, cerebral vesicle; *Ep*, epidermis; *G*, gut; *Mes*, mesenchyme; *Mus*, muscle; *Not*, notochord; *SC*, spinal cord.

in the anterior embryos was often greater than two and the amount of pigment was greater than in whole tadpoles. The numbers of pigment cells range from one to nine, the average being 3.8 for the anterior half embryos. The *a*4.2 + *A*5.1 or *A*5.2 combinations also regularly produce neural tissue. Supernumerary sensory cells may also appear in

these embryos. The sensory cell production in *a*4.2 + *A*5.1 or *A*5.2 embryos may be contrasted with that of embryos whose animal material comes from the posterior region, *b*4.2 + *A*5.1 or *A*5.2. The *a*4.2 material has responded positively in twelve of thirteen cases, whereas the *b*4.2 material gave a negative response in eight of nine cases. This comparison is of embryos from the same batches of eggs. The response of posterior animal cells is somewhat better when the inductor cells are the *A*4.1 cells of the eight-cell stage. In this case both *A*5.1 and *A*5.2 materials are represented. The positive responses with *b*4.2 + *A*4.1 were fifteen of fifty-six. This is in spite of the fact that most of the *b*4.2 + *A*4.1 operations were performed before the introduction of the semi-sterile technique.

Not only do anterior animal cells respond more often than posterior animal cells to the same inductor, but also the anterior cells produce more sensory structures. Never have the posterior animal cells produced more than two sensory cells. The number is usually one.

A further result obtained from the transplantation experiments is that the addition of posterior cells to combinations which alone would produce neural material decreases the frequency of its appearance. When the *a*4.2 cells alone were in combination with *A*5.1 or *A*5.2, twelve of thirteen embryos contained sensory cells. When *b*4.2 + *a*4.2 cells were host to *A*5.1 or *A*5.2, only seven of thirteen produced sensory cells. Similarly, when the 2*B*4.1 material was added to a 2*b*4.2 + 1*A*4.1 combination, there were no sensory cells produced in eight cases. Alone the 2*b*4.2 + 1*A*4.1 combination had been shown to form sensory cells in three of nine cases.

Although in some of the individual experiments the cases are too few, the combined data seem to allow the following conclusions: (1) Anterior animal cells have greater potency to form cerebral structures than do posterior animal cells. (2) Posterior cells tend to inhibit the formation of sensory structures in embryos containing competent materials.

Discussion

The classical works of Conklin (1905*a*, 1905*b*) on *Styela* demonstrated that early in development there is a segregation of oöplasmic materials. These visible cytoplasmic materials are correlated in normal development with particular embryonic organs or regions. However, some of these substances may be centrifugally displaced and come to lie in foreign organs (Conklin, 1931). In a sense, the segregation of visible oöplasmic materials is differentiation. Further,

isolated blastomeres differentiate in respect to cleavage patterns. But differentiation also includes the establishment of the various functional structures. The present work indicates that the anterior vegetal region is necessary for this latter type of differentiation.

The earlier idea that the ascidian egg is a strict mosaic has been altered in recent years. Schmidt (1931) has found that lateral half embryos of *Ciona intestinalis* and *Phallusia mammillata* may sometimes form the normal three adhesive papillae. Cohen and Berrill (1936) obtained some rather normal appearing larvae from lateral half embryos of *Ascidiella aspersa*. They, however, interpreted the regulation as a mechanical regulation of an original mosaic pattern. Recently, von Ubisch (1938) has described a case in which two fused two-cell embryos of *Ascidiella aspersa* regulated to form a single individual.

Dalcq (1932, 1938) has shown that lateral, or animal, or vegetal portions may be removed from the egg before fertilization without resulting depletion of organs in the larvae which develop from the egg. Reverberi (1931) obtained larvae very similar to normal larvae from fragments of fertilized *Ciona* eggs. The results of Dalcq and Reverberi plainly show that the egg is not a determined mosaic before completion of the first cleavage.

Tung (1934) suggested the possibility that adhesive papillae and sensory cells might be dependent upon extrinsic factors, since they did not form in the isolated presumptive cells. The present work indicates that induction of organs is more general in the ascidian embryo. It appears that all cells outside of the anterior vegetal region differentiate dependently. This anterior vegetal region, presumptive for notochord, spinal cord, endoderm and some mesoderm, is similar in function to the corresponding region of the amphibian embryo, the organizer region. It is capable of self-differentiation and supplies necessary developmental factors to other regions. The great difference between amphibian dorsal embryos (Ruud, 1925) and ascidian anterior embryos is that the former regulate and form more than they would as parts of intact embryos, while the latter offer no evidence of regulation.

The recent work of Reverberi (1937) demonstrates that both animal and vegetal materials must be present in egg fragments of *Ciona intestinalis* in order that the sensory cells may differentiate. Reverberi's work and the present work suggest a possible interpretation.

There are fundamental regional differences in the egg. Materials necessary for the differentiation of endoderm and notochord and for the production of inducing substances are in highest concentration in the anterior vegetal region. Materials which react with the cerebral inducing substances, or materials which produce the reacting sub-

stances, are more concentrated in the animal region, especially the anterior animal region. The contiguity of original animal and vegetal regions established during gastrulation enables the interaction of anterior vegetal inducing substance or substances and the reacting animal material, which process leads to the differentiation of cerebral vesicle.

Conclusions

1. Blastomeres from the animal region of the eight-cell stage are incapable of self-differentiation.
2. Posterior blastomeres of the four-cell stage are also unable to self-differentiate.
3. The anterior vegetal blastomeres of the eight-cell stage are capable of self-differentiation.
4. The anterior vegetal region is necessary for the differentiation of other regions.
5. The cerebral inductor is confined to the anterior vegetal region.
6. Presumptive epidermis may form brain under the influence of the inductor.
7. Anterior animal cells have greater potency to form cerebral structures than do posterior animal cells.
8. Posterior cells inhibit the formation of cerebral structures in embryos containing competent materials.

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ANDROGENETIC DEVELOPMENT OF THE EGG OF RANA PIPIENS[1]

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INTRODUCTION

The aim of the investigator in seeking to initiate androgenetic development is to remove or inactivate the female pronucleus, at the same time leaving undisturbed the male pronucleus (if it is within the egg), the cytoplasm, and conditions essential for activation and first cleavage. To achieve this, especially by mechanical means, it is important that the egg be large, that the position of the egg chromatin be detectable, and that development proceed under laboratory conditions. It is, therefore, not surprising that the amphibian egg has been generally used.

G. Hertwig, in 1911, treated the eggs of *Rana fusca* with radium emanations, then fertilized them, and obtained androgenetic development for what appears to be the first time. Since then a variety of methods have been used to remove or inactivate the egg nucleus (see below). These have been applied to various European species of frogs (G. Hertwig, 1911; P. Hertwig, 1923; Dalcq, 1932) and toads (G. Hertwig, 1913; P. Hertwig, 1923), to various species of *Triton* (P. Hertwig, 1916, 1923; G. Hertwig, 1927; Curry, 1931, 1936; Baltzer, 1933; Baltzer and de Roche, 1936; Hadorn, 1934) and to one American species, *Triturus viridescens* (Kaylor, 1937).

None of these experiments has produced an adult haploid. In general, with androgenetic haploids as with haploids produced by parthenogenesis, gynogenesis and merogany, development ceases after a few days or in some cases a few weeks, is always abnormal, and where it continues to the larval stages produces an animal which is inactive and edematous.

Despite their abnormalities, these haploids offer numerous possibilities for the study of nucleo-cytoplasmic relationships. Indeed, the abnormalities in themselves are not without interest, for an experi-

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mental demonstration of their cause should throw considerable light on the problems of differentiation. To be most serviceable as an experimental material, it seems essential that the haploids and the methods by which they are produced should possess certain positive characteristics. Their development should be fairly normal and continue to an advanced stage of differentiation; the peculiarities of haploid development should be uniformly displayed by all animals; the haploid nuclear condition should remain unchanged; and the operative technique should be simple, effective, and capable of producing relatively large numbers. Haploids produced from eggs of various species of amphibia and by a variety of methods have satisfied these criteria to varying degrees, in no case perfectly. In view of this fact it is important to experiment further with new materials and methods.

The report which follows presents the results of such experiments. An effective technique for the removal of the egg chromatin from the egg of *Rana pipiens* is described; the development which results from these operated eggs is described and compared with the normal diploid; it is shown that the great majority of these animals develop as haploids; and certain cytological observations are presented which are of possible importance in explaining the abnormalities of haploid development.

I should like to express my sincere gratitude to Professor Leigh Hoadley for his aid and advice during early investigations of this material. I am also indebted to Professor G. Fankhauser for valued suggestions in more recent studies.

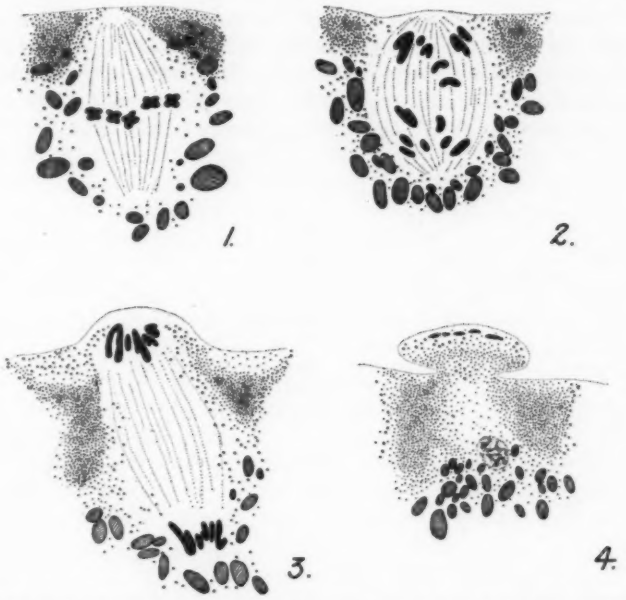
Materials and Methods

The eggs of the frog, *Rana pipiens*, secured from the state of Vermont were used in these experiments. Ovulation was induced by injecting water extracts of the anterior lobe of the frog pituitary following in general the method described by Rugh (1934). Such eggs when inseminated usually give a high percentage of fertilization and since the development which follows is perfectly normal there is little reason for considering the eggs so obtained as inadequate for experimental purposes.

The operation, which results in the removal of the maternal chromatin, is simple and effective. Since it is in part original to these investigations and since its successful application depends on an understanding of events taking place within the egg, a rather complete description follows.

At the time of insemination the egg of *R. pipiens* has undergone the first maturation division and the second division is in metaphase

awaiting the entrance of the sperm before continuing in the production of the second polar body and the female pronucleus. Sections through the egg in this stage of maturation reveal the relation of the spindle to the egg surface. (Fig. 1). It is to be noted that it lies close to the surface and is almost completely covered over by pigment granules. As the second maturation division proceeds this relationship is altered.



FIGS. 1–4. Semi-diagrammatic representations of four stages in second polar body formation of *R. pipiens* eggs. Drawings were made with camera lucida and give exact distribution of pigment granules, yolk platelets and chromosomes, only part of which are shown. Selected from considerable material sectioned at 10μ. (Eggs inseminated and kept at 12° C.) 1125 ×.

Fig. 1. Division spindle as in egg at time of insemination.

Fig. 2. Anaphase of maturation division. Stage at which spindle can be seen from exterior of egg as small black dot. Egg fixed 35 minutes after insemination.

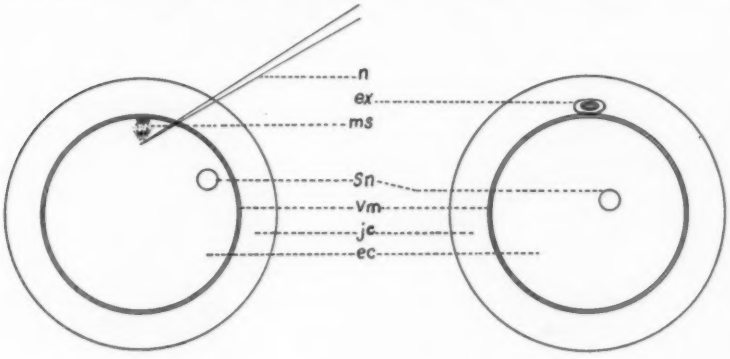
Fig. 3. Early telophase. Egg fixed 50 minutes after insemination.

Fig. 4. Polar body just forming. Egg fixed 56 minutes after insemination.

Between 20 and 35 minutes after insemination the metaphase gives way to anaphase and the pigment granules directly over the division figure become widely dispersed (Fig. 2). If, at this time, the egg is observed from the exterior under strong illumination and a magnification of more than 25 or 30 diameters the location of the maturation spindle can be detected as a small black dot. This appearance is

doubtless due to the absence of light-reflecting pigment granules over the spindle (Figs. 2 and 3). Many of these so-called black dots have been watched and in all cases they have been observed to disappear gradually (between 35 and 45 minutes after insemination) and to be replaced by the small second polar body (Figs. 3 and 4).

The removal of the egg chromatin is accomplished by means of a glass needle possessing a very fine but rigid point. While the location of the maturation spindle is apparent the point of the needle is inserted through the jelly capsule and into the cortex of the egg to one side of and diagonally beneath the spindle (Fig. 5). A slight upward motion of the needle then produces a small exovate which contains the spindle and consequently all of the maternal chromosomes (Fig. 6). When



5. 6.

FIG. 5. Diagram of operative procedure. *n*, needle; *ms*, maturation spindle; *sn*, sperm nucleus; *vm*,vitelline membrane; *jc*, jelly capsule; *ec*, egg cytoplasm.

FIG. 6. Diagram of egg and exovate after operation. *ex*, exovate; others as in Fig. 5.

the operation is performed slowly and carefully the small pellucid spindle can occasionally be seen in the yolky cytoplasm which comes out with the needle. Thus the egg is left otherwise intact with only the male chromatin present to influence the development which follows. The exovate which forms outside the vitelline membrane is soon completely detached from the egg and generally no mark remains on the embryo to mark the place of exovate origin and former attachment.

The usefulness and value of such an operation are in part determined by the ease with which it can be executed and therefore the

number of eggs which can be treated in a short length of time. Within the 10 to 15 minutes during which the maturation spindles on a group of eggs are apparent it is possible to operate on 25 or 30 eggs and exercise considerable care in so doing. If the eggs are inseminated in small quantities and at 15-minute intervals this number can be increased several times and sufficient material is made available for quantitative studies of a physiological as well as morphological character.

The loss of the small amount of egg cytoplasm which forms the exovate appears to have no harmful effect upon later development. Evidence for this statement is drawn from the following sources: (*a*) Experiments have been performed in which small exovates were produced on eggs in the immediate vicinity of, but not including, the maturation spindle. These developed normally as far as could be observed from external appearances and certainly displayed none of the abnormalities characteristic of haploid embryos. (*b*) Occasionally a normal appearing embryo arises from an operated egg (possibly as result of unsuccessful operation). In two cases these have been allowed to develop and have ultimately metamorphosed without showing any notable deficiencies. Therefore, it seems justifiable to conclude that the abnormal characteristics of the animals which result from these operated eggs are due to an altered nucleus rather than to an altered cytoplasm.

Various other methods have been applied to amphibian eggs to bring about androgenesis. The egg chromatin has been rendered inactive by radium emanations and x-rays (G. Hertwig, 1911, 1913, 1927; P. Hertwig, 1916, 1923; Dalcq, 1929, 1932), it has been removed by pricking the egg of *R. esculenta* with heated and unheated needles (Dalcq, 1932), and it has been destroyed with a needle and then withdrawn by a micropipette (Curry, 1931, 1936; Baltzer, 1933; Baltzer and deRoche, 1936; Hadorn, 1934; Kaylor, 1937).

A comparative evaluation of these various methods should be made only by one who has tried them all. Furthermore, for different eggs, different operations may be required. For example, with the egg of *Triturus viridescens* it is necessary to use a micropipette to remove the egg chromatin for an exovate is not formed by merely pricking the egg. Therefore, whatever may be the merits or drawbacks of these various methods, it is necessary in any evaluation to consider them in conjunction with the egg to which they are applied.

Further comment should be given to Dalcq's method of pricking the egg with heated and unheated needles. It is similar to the technique applied in these experiments to the egg of *R. pipiens* but from his description it does not appear that he observed the exact location of

the maturation spindle. Instead, he pricked the egg in the lighter region in the centre of the animal pole where the maturation figure is generally, but not always, located. That he did not always remove the egg chromatin, as he himself suggests, is further indicated by the presence of 5 diploid embryos in a group of 22 which developed from operated eggs.

In all experiments to be reported, experimental animals and controls were from the same female, were inseminated simultaneously, were kept under identical conditions of temperature (generally constant to $\pm$ 0.05° C.), volume of water per animal, water change, etc. For fixing, a corrosive sublimate, acetic, formalin mixture was generally used. This has been found to be especially valuable for the younger, yolky stages for it has little hardening effect. Harris haematoxylin has been found most serviceable as a general stain. With it the nuclei stain a deep blue and the yolk granules remain a purple, thus permitting some degree of differentiation.

Observations

The Development of Androgenetic Embryos

The description of androgenetic development which follows is taken from observations on several groups of experimental animals. Developmental rates and illustrations (Figs. 7–22), however, refer to one particular group (Exp. 38–1) numbering 52 experimental animals which were raised at 19.4° C. From this group and one other, experimental animals were selected and fixed at 24-hour intervals as recorded in Tables II and III. Controls were simultaneously preserved. In this way material was provided for an examination of internal as well as external morphogenesis. While some variation is shown among the members of a single group, especially in the older stages, there is a majority which show the general features described below.

Observations were normally begun at the time of first cleavage. This may take place anywhere between 2 and 3 hours after insemination depending on the temperature at which the eggs have been inseminated. It is customary for between 90 per cent and 100 per cent of the operated eggs to divide normally and to do so simultaneously with the control eggs (Table I). This behavior, while typical for these androgenetic frog eggs, is not typical for all amphibian eggs. For example, Kaylor (1937) reports that a considerable proportion of his androgenetic *Triturus viridescens* eggs cleaved abnormally or failed to cleave at all and that 90 per cent of those for which cleavage records were available showed a significant delay in the appearance of the first cleavage furrow.

Blastula development of androgenetic *R. pipiens* embryos is quite normal. As the time for gastrulation approaches slight indentations occasionally appear in the animal hemisphere of the blastula. Since these later disappear, do not occur in all of the experimental embryos, and have been noted in the controls, they are not considered a typical abnormality of androgenetic development. Comparative examination of androgenetic and control late blastulae reveals noticeably smaller cells in the former. This observation made from the outside has been verified from sections. Though cell counts have not as yet been made, it seems probable that there are more cells in the androgenetic blastulae and that they have resulted from a more rapid rate of cell division.

Gastrulation begins approximately one hour earlier in the controls than in the experimental animals. This constitutes the first clear

TABLE I

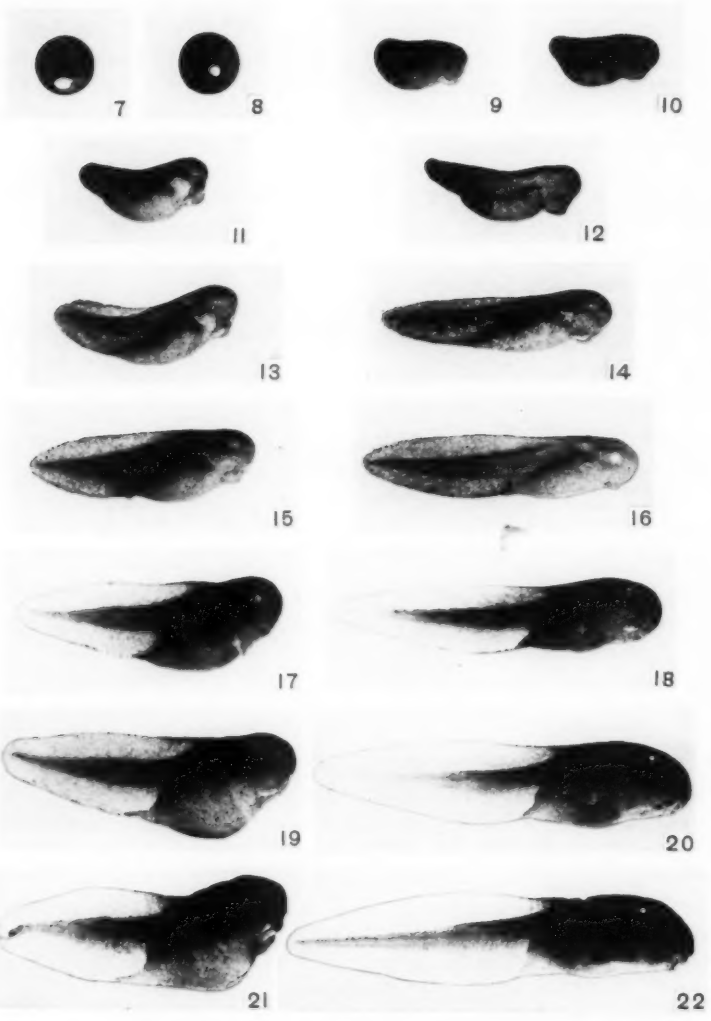
Record of first cleavage in several lots of operated eggs.

Exp. Number	Number of Eggs Operated	Number that Cleaved	Percentage Cleavage
36–5	38	38	100
36–10	179	179	100
36–13	278	255	92
37–2	38	38	100
37–4	70	69	98
38–1	52	52	100

Note: Experiments 36–10, 37–2, 37–4, and 38–1, provided the data upon which this report is based.

indication of a retardation of differentiation. This delay is a distinct characteristic of amphibian androgenetic development and has been reported by other investigators for a variety of species. It is more clearly indicated at the end of 24 hours by a difference in the size of the crescentic blastopore (smaller in the androgenetic embryos). In the great majority of the experimental animals gastrulation proceeds normally and by the end of 48 hours the yolk plug stage is reached (Figs. 7 and 8). The larger yolk plug of the androgenetic embryo provides evidence of retarded development.

During the formation of the neural tube on the third day it becomes clearly evident that androgenetic development is not simply normal development, slightly delayed, but is abnormal as well as delayed. For instance, the neural plate of the experimental animal remains approximately one-third shorter than the neural plate of the control; the neural folds stand up less prominently from the body of



FIGS. 7–22. Photographs of typical androgenetic embryos and normal diploid controls from a group of operated eggs (Exp. 38–1) raised at 19.4° C. Figs. 7, 9, 11, 13, 15, 17, 19, 21 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old androgenetic haploids. Figs. 8, 10, 12, 14, 16, 18, 20, and 22 are respectively 2-, 3-, 4-, 5-, 6-, 8-, 10-, 12-day-old controls. Photographs are of fixed animals. *ca* 5×.

the embryo, and the neural groove is more shallow. There are probably indiscernible abnormalities in gastrulation which contribute to the above and in turn to the more pronounced departures from the normal shown by the older animals. Closure of the neural folds is completed between 2 and 3 hours later than in the controls which indicates an increasing delay in differentiation.

The 3-day-old experimental animal depicts abnormal as well as delayed differentiation (Figs. 9 and 10). The tail-bud is shorter, the abdomen remains abnormally large and round, and the head is smaller and apparently less differentiated. From the third day on development is characteristic of the androgenetic embryos only, and exact stages for stage comparisons with the controls are no longer possible.

The typical 4-day-old experimental animal is smaller than the control, shows a pronounced bend in the back, a shorter and round abdomen, and a head which does not show the normal downward bend or cranial flexure (Figs. 11 and 12). The first indications of gill filaments which appear at this time in the control do not appear in the androgenetic animals until almost a day later.

Certain of these abnormalities persist on the fifth day and are clearly shown in Fig. 13. The 5-day-old control possesses a pulsating heart and a complete gill circulation whereas the experimental animals do not clearly show these features until the end of the sixth day.

In the typical 6-day-old experimental animal (Fig. 15) the back has straightened but in total length the animal is still considerably shorter than the control. It is of interest to note that the head of this animal (Fig. 15) more closely resembles that of the 5-day-old control (Fig. 14) than it does the 6-day-old (Fig. 16). But even in this similarity there are discrepancies as indicated by the position of the olfactory pit. Generally more than one-half of the androgenetic embryos of this age show a pulsating heart and of these fully one-third can be expected to have a fairly normal gill circulation.

It is typical for a few of the 7-day-old animals to become edematous and with each day thereafter the number of edematous animals increases. This condition may become so extreme that not only the body cavity but also the tissue spaces in the head become filled with fluids (Fig. 21). When this extreme is reached death generally ensues. Therefore, if the animals are to be saved, fixing agents are applied. In the group of animals from which this description is illustrated most of the animals were fixed on the ninth and tenth days (Table II).

During the eighth day the operculum grows over whatever gill filaments the animal may happen to have. This operculum development is outstanding in that it takes place at the same time and rate

as in the controls whereas other organs may be more than 24 hours delayed.

TABLE II

A record of fixation and examination for chromosome numbers of animals which developed from 52 operated eggs. (Temp. 19.4° C.)

Age at Time of Fixation	Number Fixed	Classification
1 day	3	3 haploids
2 days	4	2 haploids 1 normal diploid 1 abnormal diploid
3 days	5	3 typical haploids 2 atypical haploids
4 days	2	2 typical haploids
5 days	1	1 haploid
6 days	3	1 typical haploid 2 atypical haploids
7 days	2	2 typical haploids
8 days	1	1 haploid
9 days	11	11 edematous haploids, 5 of which show a few diploid nuclei
10 days	8	8 edematous haploids, 7 of which show a few diploid nuclei
11 days	3	3 edematous haploids, 2 of which show a few diploid nuclei
12 days	6	4 edematous haploids showing a few diploid nuclei 1 edematous haploid with several diploid nuclei 1 diploid-triploid, developed more successfully than the typical haploid
15 days	1	1 haploid-diploid, haploid on one side, diploid on other side.
22 days	2	1 pure diploid of normal structure 1 triploid, appearance of normal diploid

Summary: 46 haploids, 3 diploids, 1 triploid, 1 haploid-diploid, 1 diploid-triploid; 89 per cent of population haploids.

After the eighth day there is slight change in the gross appearance of the experimental animals except that the majority become increasingly edematous (Figs. 19 and 21). Differentiation of some

parts continues but a discussion of such differentiation is not essential to this general description. It should be mentioned, however, that in those cases where a circulation is established, at least for a short time, differentiation is more successful and the animal lives over a greater number of days.

The behavior of these animals can scarcely be called normal. Most of the time they are rather inactive and lie on their sides on the

TABLE III

A record of fixation and examination for chromosome numbers of animals which developed from 38 operated eggs. (Temp. 20.1° C.)

Age at Time of Fixation	Number Fixed	Classification
2 days	2	2 typical haploids
3 days	1	1 typical haploid
4 days	2	1 typical haploid 1 slightly atypical haploid
5 days	2	2 typical haploids
6 days	1	1 typical haploid
7 days	10	1 typical haploid 8 edematous haploids 1 very atypical haploid
8 days	4	4 edematous haploids
9 days	11	10 edematous haploids, 5 of which show a few diploid nuclei 1 accidentally destroyed had developed as haploid
10 days	5	1 edematous haploid 2 edematous haploids showing a few diploid nuclei 1 haploid-tetraploid (Fig. 32) 1 died before fixation, had developed as haploid

Summary: 37 haploids, 1 haploid-tetraploid; 97 per cent of population haploids.

bottom of the container. When sufficiently stimulated, however, they will respond by swimming about in undirected circles.

Chromosome Numbers and Nuclear and Cell Size

That the embryos which develop from operated eggs are haploids has been indicated, not only by the rather certain removal of the second polar spindle, but also by the abnormalities which they show. For further evidence, however, a cytological examination was made of some part or parts of each animal of two different groups of operated

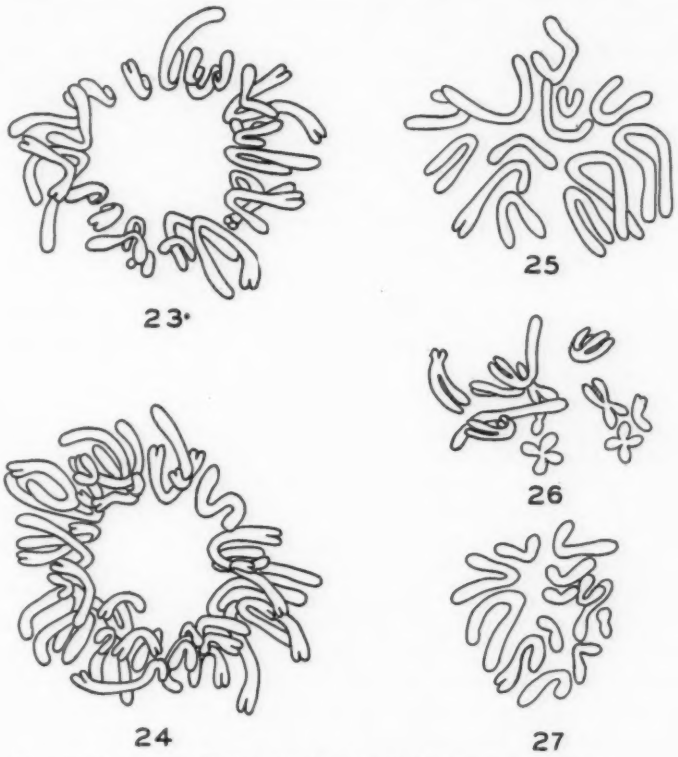
eggs. For the younger animals this evidence was obtained from sections; for the older animals, from tail tips clipped from fixed specimens and made into whole mounts. In the case of each animal one or more metaphase plates were examined in detail to establish the chromosome number, and, in addition to this, a record was kept of the total number of division figures which could be identified as haploid or otherwise by brief examination only. In general, the quality of the preparations permitted the examination of 25 or more (in some cases many more) mitotic figures. The results of these studies are summarized in Tables II and III and additional evidence is shown in Figs. 23 to 27.

It is clearly shown that the vast majority of these operated eggs developed as haploids. For the exceptions there is at the present time no definite explanation. There always remains the possibility that they resulted from unsuccessful operations whereby the egg chromatin remained within the egg. But even if this is the explanation, the results indicate that at its worst the method is about 90 per cent effective. The animals which did not develop as haploids were easily detected for they showed either the characteristics of normal diploids or other characteristics not typical for haploids.

It is of interest to compare these results with those reported by Dalcq (1932) for androgenesis with the egg of *R. esculenta* and by Parmenter (1933) for parthenogenesis with the egg of *R. pipiens* and *R. palustris*. Out of 22 operated eggs in Dalcq's experiments 5 developed as diploids; out of 29 embryos which developed parthenogenetically Parmenter reports 10 pure diploids. These results would lead one to expect a larger number of diploids among these androgenetic *R. pipiens* embryos than have been found. In the case of Dalcq's results, however, the high percentage may be due to a poor localization of pricking and not to any marked instability of the frog haploid nucleus. But failure of operative technique could scarcely account for the large percentage of parthenogentic diploids. Several explanations, which are reviewed by Parmenter, have been suggested. It is possible that a study of very early cleavage stages will provide an explanation for this difference between the results of parthenogenetic and androgenetic experiments.

It has been noted (Tables II and III) that the tail tips of some of the older haploids show a few diploid nuclei. These were identified by their larger size and by the presence of two nucleoli (Fig. 32). Since they occur solely within the tissues of haploids which have more or less reached the end of their development, it would seem that some condition or conditions within these animals are related to their origin. But as to the mechanism of their origin, there is only slight evidence.

In a very few cases monastral divisions of haploid nuclei have been observed. The presence of scattered diploid nuclei in the older stages is not a feature confined solely to these androgenetic haploids.



FIGS. 23–27. Camera lucida drawings of mitotic figures. 3250 ×.

Fig. 23. Diploid metaphase from tail epithelium of 15-day-old control.

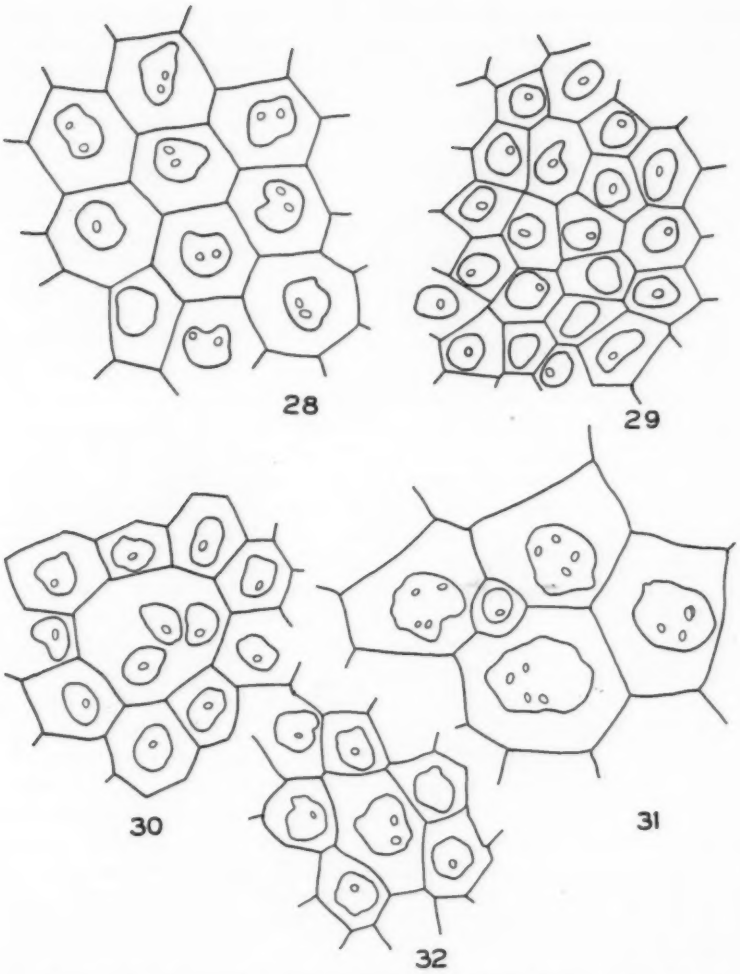
Fig. 24. Triploid metaphase from tail epithelium of 22-day-old triploid animal which developed from an operated egg (Table II). Shows 36 chromosomes (triploid 39).

Fig. 25. Haploid metaphase from 1-day-old androgenetic haploid in early stages of gastrulation.

Fig. 26. Haploid late prophase from tail epithelium of 7-day-old androgenetic haploid.

Fig. 27. Haploid metaphase from cell in tail mesoderm of 10-day-old androgenetic haploid.

Dalcq discovered the same in his preparations and Parmenter located a few diploid divisions in the tissues of some of his older animals which were otherwise predominantly haploid.



FIGS. 28–32. Camera lucida drawings of cells and nuclei from tail epithelia. 750 ×.

Fig. 28. From 9-day-old diploid control.

Fig. 29. From 9-day-old androgenetic haploid.

Fig. 30. From 9-day-old androgenetic haploid; shows 3 haploid nuclei in one large cell.

Fig. 31. From 10-day-old androgenetic embryo showing large tetraploid nuclei and cells which predominate epithelium on one side of tail (Table III).

Fig 32. From 10-day-old androgenetic haploid showing diploid nucleus and cell among haploid nuclei and cells.

The nuclear and cell size in haploids has been repeatedly shown to be smaller than in diploids and to this rule these androgenetic frog haploids are no exceptions (Figs. 28 and 29). Observations on haploids and diploids of all ages reveal that this relationship holds whether the observed animals are one day or several days old. It has also been noted that with an increase in chromosome number to triploid and tetraploid there is a corresponding increase in nuclear and cell size (Fig. 31).

There is a tendency in these haploids for several nuclei (as many as seven have been counted) to occupy a single cell. With this increase in number of nuclei, as with an increase in chromosome number, there is a corresponding increase in cell size (Fig. 30).

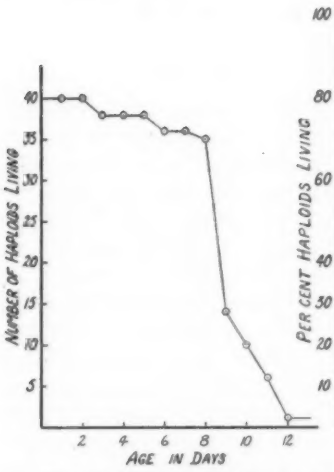
The Extent and Uniformity of Development

Studies of groups of androgenetic embryos involving the fixation of representative types at regular intervals do not indicate accurately the extent or uniformity which might be displayed by a total population of such animals. A simple demonstration of these qualities was obtained by allowing each member of a given population of 40 animals to proceed as far as possible in its development. These animals were kept in separate containers under uniform conditions (temperature constant at 20.1° C.). While they were ultimately killed by fixing agents, the same were not applied until the indications were very definite that life would not continue for many hours. The graph presented in Fig. 33 summarizes the data of this experiment. As can readily be seen, up until the fifth day all but two of the original animals were living. From this time until the end of the eighth day there was only a slight change. At this time, however, it was necessary to preserve a large number of them because of their extreme edema. After this pronounced drop the decline is more gradual until the eleventh day after which only one animal remained alive. This one continued to live for several weeks, but, as was expected, it proved to be part haploid and part diploid. The other 39 animals were considered as haploids on the basis of the development which they displayed.

Since these animals were killed by artificial means it seemed advisable to examine some data from earlier groups of androgenetic embryos which had been raised at temperatures averaging 20° C. and in which death was caused by natural agents rather than fixing agents. The data are summarized in Fig. 34 and it is clearly evident that there was a sharp increase in the mortality rate after eight days just as depicted in Fig. 33. Hence the first graph (Fig. 33) can be considered as a correct representation of the survival value of a

population of androgenetic *R. pipiens* haploids raised at a temperature of 20° C.

It should not be concluded from these results that androgenetic frog embryos are incapable of further development than that expressed by a 10 or 12-day-old animal raised at 20° C. It is certainly true that the vast majority never go beyond this stage, but the occasional animal will continue longer and while showing abnormalities and a slower rate of growth, it will nevertheless take food and live over several weeks or months. Two animals of the group described in Fig. 34 lived for five weeks and another, which developed from an



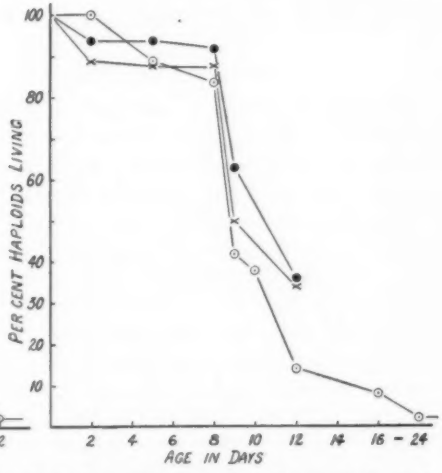


FIG. 33. Graph depicting survival of population of 40 androgenetic haploids which were fixed when it was judged that they could not survive many hours.

FIG. 34. Graph depicting survival of 3 different populations of androgenetic haploids which were allowed to die of natural causes.

operated egg in more recent experiments, lived for sixteen weeks. It developed into a sizeable tadpole with small hind limbs. Cytological examination of the tail epidermis has revealed that it was predominantly haploid.

The uniformity of a group of androgenetic embryos cannot, unfortunately, be measured by any known unit but must be left entirely to the judgment of the investigator. The fact that the majority of the animals live for eight days, suggests that early development is quite normal and uniform from animal to animal. If, on the other hand, a few animals had died each day and in all stages from cleavage to tadpole, the development could be referred to as un-uniform. The

individual animals of the group, the survival of which is described in Fig. 33, were examined every day throughout the duration of the experiment and by means of this examination were compared with one another and with one of the group selected as type. From this study the uniformity can be described as follows: until the end of the third day it was practically perfect, from the third to the fifth day it was fair and from the fifth day on it was rather poor, with differences becoming more pronounced. In other words, as the complexity of structure increased the uniformity of the population decreased.

It is difficult to compare the success (extent and uniformity) of androgenetic development displayed by these *R. pipiens* with the same development of other species. Investigators have used ages rather than stages to describe their results and in using such a unit as days-development, temperature variations become important. Among species of frogs, the androgenetic development described by G. Hertwig (1911) for *R. fusca* and by Dalcq (1932) for *R. esculenta* is no more successful than that reported here for *R. pipiens*. In fact, as far as uniformity is concerned, the results with *R. pipiens* seem to be better. This may be due to the method of operation rather than the species of egg. It has been stated that toad haploids develop better than haploids from the larger frog eggs and that *Triton* haploids develop better than the anurans (P. Hertwig, 1923). A comparative study of amphibian haploidy made at the present time might produce cause to qualify this statement. Until the haploid development of a greater variety of amphibian eggs has been studied it will be impossible to determine whether it is the species of egg, the egg size, the method of initiating haploid development or some combination of these or other factors that makes for greater success in some cases than in others.

Internal Morphology and Development

The typical experimental animals fixed at various ages as recorded in Tables II and III have been sectioned. The description which follows is based on an examination of these sections.

Observations on internal morphogenesis support those on external in showing that development is delayed and abnormal. These facts can be illustrated by an examination of eye development in 3-, 4-, and 5-day-old haploids and controls (Figs. 35–40). In the 3-day-old diploid (Fig. 35) the optic vesicles have extended to the head ectoderm and are in a position to induce lens formation. In the haploid (Fig. 36) the vesicles are smaller, have scarcely reached the head ectoderm and therefore show delayed development. By the end of four days, the control (Fig. 37) shows a well-formed optic cup and lens whereas the haploid (Fig. 38) has advanced only slightly beyond the stage repre-

sented by the 3-day-old control and shows only the beginning of lens formation. The 5-day-old haploid (Fig. 40) compared with the control of the same age (Fig. 39) shows an optic cup which is decidedly abnormal. Its dorsal half and the lens are quite similar to the same structures in the 4-day-old control (Fig. 37), but the ventro-lateral lips of the cup fail to grow out leaving a wide choroid fissure. It looks as if the optic stalk in failing to elongate had held in the ventral portion of the cup. Later development does not make up this deficiency in the optic cup, and by a continued proliferation of cells in the retinal layer the structure becomes increasingly abnormal. Only rarely is development more nearly normal. Thus it is observed that while development makes a fairly normal beginning as shown by the vesicle of the 3-day-old, the results as indicated by the 5-day-old and older stages are quite abnormal.

The following survey presents some further outstanding features of haploid internal morphology and morphogenesis as observed from sections of the older stages. They represent observations on the typical haploid.

Nervous System.—An examination of the anterior central nervous system reveals in the oldest haploids a poorly developed brain. In many cases the ventricles are almost entirely obliterated by a marked proliferation of cells or nuclei and a resulting thickening of the brain walls. This condition continues to the posterior end of the medulla. The spinal cord, on the other hand, displays a persisting neurocoele and in the caudal regions is a relatively normal structure. The nuclei are more numerous than in the diploid and in the sections of the older haploids they give way to a vacuolar type of picnosis. The fibre tracts are always indefinite in limitations and have nuclei scattered through them in an abnormal fashion.

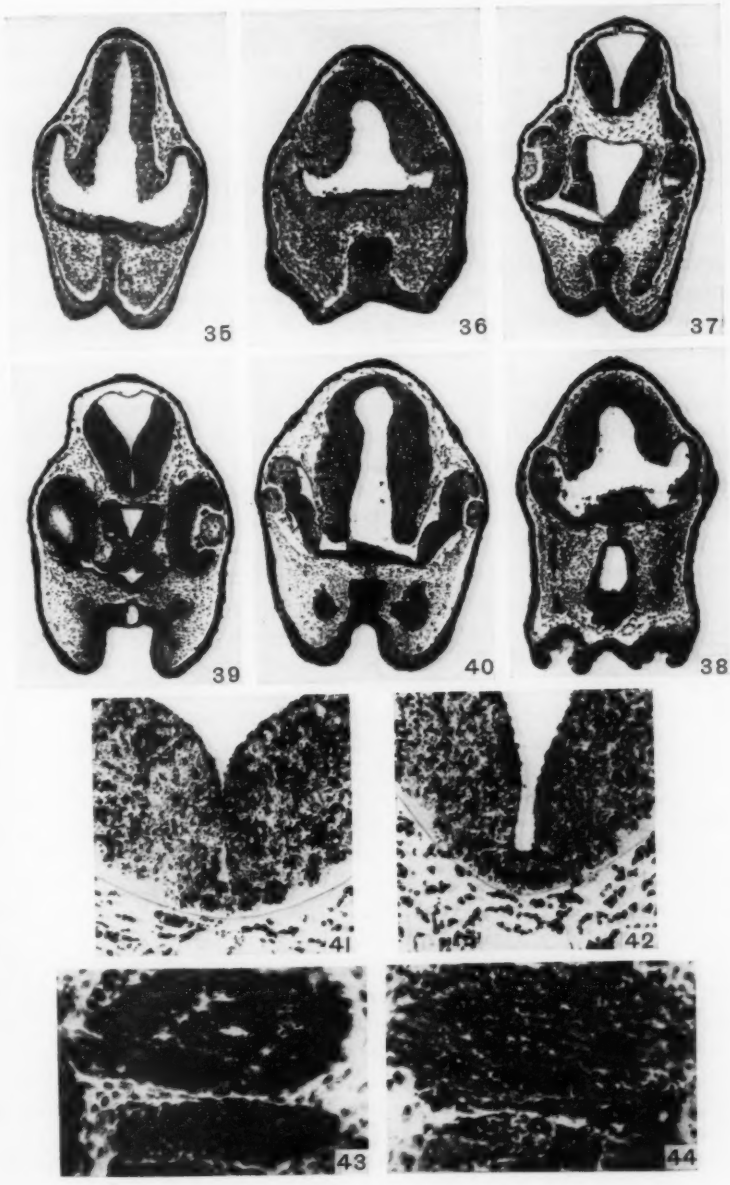
The eye develops abnormally as indicated above. Lenses are absent in many cases and when present are considerably smaller than normal.

FIGS. 35–40. Photomicrographs of sections through optic vesicles and optic cups of haploids and controls, aged 3, 4 and 5 days. 38 ×.

Fig. 35. From 3-day-old control.
Fig. 36. From 3-day-old haploid.
Fig. 37. From 4-day-old control.
Fig. 38. From 4-day-old haploid.
Fig. 39. From 5-day-old control.
Fig. 40. From 5-day-old haploid.

FIGS. 41 AND 42. Sections through the same region of the medulla of 5-day-old control (Fig. 41) and androgenetic (Fig. 42) embryos. Yolk granules are very darkly stained inclusions. 160 ×.

FIGS. 43 AND 44. Sections through the same muscle in the pharyngeal region of 7-day-old control (Fig. 43) and androgenetic (Fig. 44) embryos. 160 ×.



FIGURES 35–40.

The otocyst, unlike the optic cup, differentiates at more nearly the normal rate, but does so abnormally. Instead of one vesicle being at first formed, several develop within the mass of cells which originally arises from the head ectoderm.

The Notochord.—In striking contrast with the nervous system, the notochord is among the best developed and differentiated structures in the androgenetic larvae. By the end of the third day it is well formed and displays a cross-sectional area approximately the same as that of the controls. This same relative size generally persists and when the cells become vacuolated they tend to be smaller and therefore more numerous than in the diploid. Whether or not the more successful differentiation of this structure is related to its early histogenesis is a question of some interest.

The Pronephric Kidney.—This appears slightly later than in the controls and shows fair development. The nephrostomes open into the body cavity and though some difficulty is encountered in tracing the course of the convoluted tubules, they appear to connect with the common duct. This latter is patent and has been traced to an open cloaca in edematous as well as in the more normal androgenetic larvae. This has its interest in that an incomplete lumen in the pronephric duct has been used to explain the edema common to these haploids (Dalcq, 1932). It is evident that such could not be the cause in all cases. The convolutions of the androgenetic kidney are less extensive than in the control kidney of the same age, which suggests a delay in elongation of the tubules. This earlier kidney is vascularized though generally to no avail as the circulation is seldom functional. Evidence for this latter fact often exists in the form of abnormal accumulations of blood cells around the tubules.

Other mesodermal derivatives such as the somites and visceral arches show fair though delayed differentiation. The somites tend to be smaller in cross-sectional area and to be underdeveloped in the thin dorsal extensions lateral to the nerve cord. The muscle cells are smaller and less compactly grouped.

The Circulatory System.—The circulatory system is functional in very few cases though the heart beats in many. The differentiation of the heart is considerably delayed and is generally about 24 hours behind the control in showing its first pulsations. The larger vessels can be located and traced, but the development of capillary connectives is doubtful. This latter failure is suggested by the patches of blood cells which accumulate in various regions of the body not normally associated with blood formation. Only in the occasional haploid can a good capillary circulation be located in any part of the body. The

blood cells are generally less numerous, are smaller, and contain more yolk granules. They often contain 2 or 3 nuclei after the yolk platelets have disappeared.

The Gut.—The gut is markedly retarded in its differentiation. This is most emphatically shown by the fact that in a 9-day-old edematous haploid the gut appears as an almost straight tube whereas in the control of the same age it is considerably coiled. The walls of this short gut are thicker and the cells are packed with yolk. The derivatives of the gut likewise differentiate rather tardily. For example, the lungs, arising from the fore-gut, are in about the same stage of development on the ninth day as they were on the seventh day in the controls. This 2-day delay in differentiation is, however, not common to the whole animal.

The Ectoblast.—The ectoblast in its differentiation more closely parallels the controls than any other part of the embryo. Oral suckers, olfactory pits, mouth parts and operculum all differentiate quite normally and at approximately the normal time. The ectoderm, at first wrinkled and thicker than in the controls, becomes thinner as the animal becomes edematous. Tumor-like proliferations of the ectoderm occasionally appear, and are not unlike those shown by frog embryos treated with weak solutions of 2,4-dinitrophenol (Dawson, 1938), or with high temperatures (Hoadley, 1937), or developed from over-ripe eggs (Witschi, 1930).

Yolk Supply

Only a brief examination of the sections of these haploids was necessary to show that yolk disappears more slowly from the cells of the haploid than from the diploid. Since it was felt that considerable importance could be attached to this observation studies of yolk content were made along with studies of morphology. These are considered but the beginning of future studies which may throw some light on the causes of haploid deformities.

Until the haploids and controls are 4 days old (20° C.) the yolk content of the cells in all regions of the embryo is so great that microscopic comparisons are without value. In animals varying from 4 to 7 days a comparative examination of the same organs in haploids and diploids of the same age reveals a greater quantity of yolk in the cells of the haploid (Figs. 41 to 44). Within these age limits this difference holds for all tissues of the embryos though it is more apparent in some than in others. In haploids older than 7 days the yolk supply of some tissues (ectoderm of 8- and 9-day-olds) is completely exhausted while in others (the gut) it is still possible to observe a greater quantity in the cells of the haploid. It can be noted further that differentiation seems

to be more delayed in regions most richly supplied with yolk. Further observations, and if possible measurements of yolk content, are necessary before it can be stated that the cells of a haploid tissue do not approach a normal stage of differentiation until their yolk supply has been reduced to the normal extent. It is hoped that future experiments may clear up this matter and provide a basis for definite conclusions.

Discussion

The results of these experiments indicate that the operation by which the maturation spindle is removed from the egg is successful. Approximately 90 per cent of the operated eggs develop as haploids, the haploid nucleus being that of the sperm. It has been shown that under the influence of this nucleus development proceeds for 8–10 days (20° C.) and produces a tadpole showing considerable differentiation. This is abnormal, however, and only future experiments on other eggs will indicate whether more normal haploid development is possible among the Salientia. The uniformity displayed by these populations of haploid embryos has been described as good over the first 3 days and fair from the third to the fifth days. This degree of uniformity appears to be a distinctive feature of this material for it is not clear that similar results have been previously obtained with other eggs and methods.

It is shown, therefore, that haploid embryos of suitable quality are made available in sufficient numbers for physiological studies and measurements. The abnormalities which they demonstrate occur in sufficient uniformity to make the study of their cause attractive and possibly productive. And, from another angle, they become particularly valuable as a material for hybridization experiments involving the mixing of the cytoplasm of one species with the nucleus of another.

The subject of special interest in connection with this report is the abnormal retarded development and reduced viability of these haploids, which, it is clear, must be related to the presence of only the haploid chromosome complement. Recessive genes, lethal or otherwise, unsuppressed by dominant allels would, if present in the sperm nucleus, find definite expression in these haploids. It seems hardly probable, however, that these would occur with such regularity within the male chromosomes as to produce, for example, a similar reduction in the length of the neural plate in almost every haploid in a population of 40 experimental animals. It is more logical to associate such a departure from the normal with the presence of a haploid nucleus within a quantity of cytoplasm normally associated with a diploid nucleus.

Several hypotheses have been proposed to account for these haploid abnormalities and Fankhauser (1937) finds in them a common

idea: a disturbance of the metabolism of the haploid cells. As to the nature of this disturbance there is no clear understanding, but it is presumably due to a supply of yolk and cytoplasm excessive for the haploid nucleus. There is some evidence in support of this hypothesis in the results of these investigations. It has been noted that the yolk supply disappears more slowly from the cells of the haploid than from the diploid. It has also been noted that differentiation is delayed and abnormal and that the delay appears to be more pronounced in tissues containing the greatest amount of yolk. From these observations it is not unreasonable to link excess yolk with delayed and abnormal differentiation. Additional supporting evidence comes from experiments on merogonic development. In the production of merogonic haploids the quantity of cytoplasm is more or less reduced and the normal karyoplasmic ratio tends to be restored. One such fragment of a *Triton taeneatus* egg developed through metamorphosis and constitutes the most successful case of amphibian haploidy on record (Baltzer, 1922; Fankhauser, 1938). Thus a decrease in egg cytoplasm to conform with the haploid nucleus may have permitted more normal development.

While these observations suggest a cytoplasmic influence as being responsible for the abnormalities, there is evidence which indicates that the influence in some cases arises from the nucleus. For instance, investigations of the early cleavage stages of merogonic egg fragments of *Triton palmatus* and *Triturus viridescens* have shown an unequal distribution of chromosomes (Fankhauser, 1932*c* and 1934*c*). This has been held responsible for the high mortality rate which it is customary for these merogonic embryos to show before or during gastrulation. The same explanation has been extended to the non-viable blastulae and gastrulae among *Triturus viridescens* embryos (Fankhauser and Kaylor, 1935). It is impossible, however, for any such alteration in nuclear structure to be responsible for the abnormalities of the typical frog haploid since a complete haploid complement of chromosomes has been observed in all cases studied.

Only when these studies have been extended and more is known concerning nucleo-cytoplasmic reactions whereby differentiation is brought about will it be possible to state with any certainty the conditions within the cytoplasm or nuclei of these haploids which make more normal development impossible.

Summary

1. A technique is described by which the second maturation spindle and so all of the maternal chromatin can be removed from the egg of *R. pipiens* following its activation and penetration by the sperm.

2. The operation as applied to this egg is considered satisfactory for a large number can be treated in a short time, the maturation spindle is removed with certainty, and the slight amount of cytoplasm removed has no destructive effect on the development which follows.

3. Between 90 per cent and 100 per cent (generally 100 per cent) of the operated eggs undergo first cleavage simultaneously with the controls, and of these the majority develop for eight days, a few considerably longer.

4. The development of androgenetic haploids compared with diploid controls of the same age is abnormal and delayed. Certain features of external and internal morphogenesis are described.

5. It is shown that 90 per cent of the operated eggs can be expected to develop as haploids. This haploid nuclear condition remains unchanged until the final stages of development and then is altered only by the presence of a very few diploid nuclei.

6. As is typical for haploids, the cells and nuclei of these androgenetic embryos tend to be smaller than those of the diploid controls.

7. The development shows a high degree of uniformity from animal to animal over the first five days after which the differences become more pronounced.

8. Yolk disappearance from the cells of the haploids is notably delayed.

9. The ease of production and the success of development of *R. pipiens* androgenetic embryos seems to provide one of the best possibilities so far encountered for the study of haploid morphogenesis from eggs which normally develop as diploids.

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THE ILLUMINATION OF THE EYE NECESSARY FOR DIFFERENT MELANOPHORIC RESPONSES OF FUNDULUS HETEROCLITUS[1]

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It has been established by previous investigations (Butcher, 1938) that the upper region of the retina of *Fundulus* is related to the paling of the body and the lower region to the darkening of the fish. During the course of these investigations many problems were encountered and left unsolved. Among them were: (1) Why does illumination of the lower region of the retina with a Mazda lamp cause most fishes to darken, but illumination of the upper region with a Mazda lamp induce only a few to pale? (2) How much of the regions have to be illuminated to elicit the related melanophoric responses? (3) To what extent does illumination of the upper region have to be eliminated in order that darkening can be induced by illuminating the lower region? (4) Is the paling response more easily elicited when light is entirely eliminated from the lower region?

The cause of a fish assuming a paler shade in a shaded white box than in a brightly lighted gray box was also investigated. It seemed that the assumption of the shade of the background by the fish might depend upon the ratio of the direct light coming from above and the reflected light from below which enters the eye as Sumner (1911), and Sumner and Keys (1929) have contended to be the case for the flounder.

The present investigations show that when a Mazda lamp is placed above fishes in a black dish, the image of the lamp falls upon enough retinal receptors in the lower region of the eye to induce darkening of the body, but when the same lamp is placed below fishes and its image falls upon the retinal receptors of the upper region, this image is not large enough to induce the paling response. Illumination of a large area of the upper region is, therefore, necessary to induce the paling response. Paling is also more easily elicited when only the upper region is illuminated. Illumination of the lower region induces darkening only when there is very little illumination to the upper region.

The melanophore response elicited by illuminating the lower region

[1] Reported before the American Society of Zoologists at the December, 1938, meetings. *Anat. Rec.*, vol. 72 (suppl. no. 4), p. 80.

of the eye may be reduced by the simultaneous illumination of the upper region. For instance, illumination from above to the lower region of the eye causes darkening of the body when there is little reflected light from the bottom of a black dish to the upper region. If a gray bottom is used, a greater percentage of the light is reflected to the upper region of the eye, a greater inhibitory reaction is induced, and the degree of darkening of the body is reduced. If this gray back-

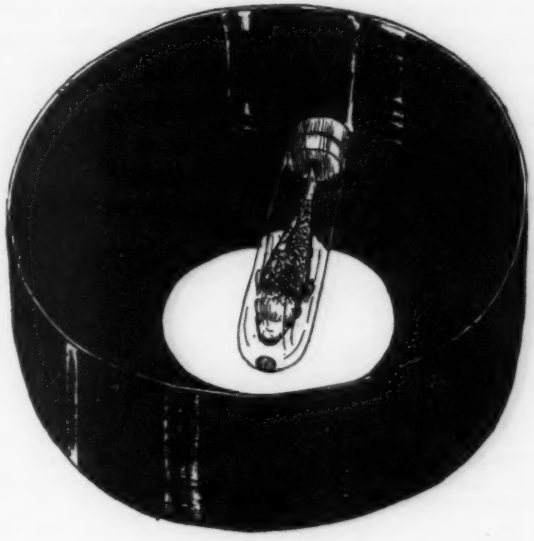


FIG. 1.

ground is more intensely illuminated from above, then the reflected light is greater to the upper region. The same ratio, however, persists between the direct and the reflected light, a proportional inhibitory effect is induced and the fish assumes the same shade as when the intensity of the direct light is lower.

The Conditions Affecting the Paling Response

The sides of crystallizing dishes, 20 cm. in diameter, were lined with black paper which reflected approximately 1 per cent of the light striking it. Fishes were placed in these dishes containing water 4 cm. deep, and the top of the dish was covered with black paper. When the fishes were illuminated through the glass bottom by a 60-watt,

inside-frosted Mazda lamp, placed 18 cm. below the dish, a few of them became slightly pale and the rest assumed an intermediate shade. The fishes, in this instance, were receiving about 200 footcandles of illumination (determined by a Weston photronic illuminometer) from the lamp while the brightness of the lamp was approximately 58,000 footlamberts.[2]

If a piece of white paper or opal glass, as large as the bottom of the dish, were inserted between the source of illumination from the Mazda lamp and the bottom of the dish, the fishes readily paled even when the brightness below them was 1 footlambert or less. The image of the white bottom being larger than the image of the bulb alone fell on a great many more retinal receptors. Paling, therefore, depends mainly upon the size of the white area seen by the fishes.

TABLE I

Relation between body size and diameter of circle below fish necessary to induce paling.

Number examined	Length of body	Width of body at level of eyes	Diameter of circles and number paling			
			9 cm.	7 cm.	5 cm.	3 cm.
6	mm. 40–45	mm. 5–6		6	6	2
59	50–60	7–8	59	48	16	0
15	60–70	9–10	15	14	7	0
7	70–80	10	7	7	0	0

As a means of determining the size of the white area necessary for inducing paling of the body, fishes were enclosed in glass tubes (16 mm. inside diameter) which had small openings at both ends for the circulation of water. These tubes with the fish inside were placed over circles of white paper in such a way that the fish's head was above the center of the circle (Fig. 1). The circles were then either illuminated from above or from below.

To induce paling of fishes 50 mm. in length, circles 7 cm. in diameter were usually necessary (Table I). When the fishes were over 3 cm. circles, an intermediate shade was always assumed. Fishes paled equally as well when the circles were exposed to 4.5 footcandles as to 450 footcandles from above.

[2] The author is greatly indebted to Mr. Frank Benford of the General Electric Company, Schenectady, N. Y. for determining the brightnesses with a Luckiesh-Taylor Brightness-Meter.

Figure 2, which is drawn to scale, shows approximately the size of the image in the upper region of the eye when the fish was over the various circles. It is evident that a large area of retinal receptors must be stimulated before paling is induced. Images of 5 and 7 cm. circles, being nearly the same size (Fig. 2), caused only slight differences in the degree of paling. It might have been better to use a square tube to hold the fish, since a round tube probably acted as a cylindrical lens, and the fish did not get an image quite like the white circle and even illumination.

Fishes of various lengths were tested in tubes of the same size over circles of white paper. Since the body of a small fish covered less of the 3 cm. circle than did the body of a large fish, the small fish saw more of the circle (Figs. 3 and 4), and assumed a paler shade over the 3 cm. circle than the large fish (Table I).

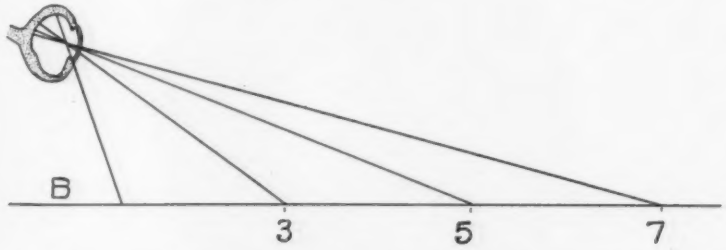


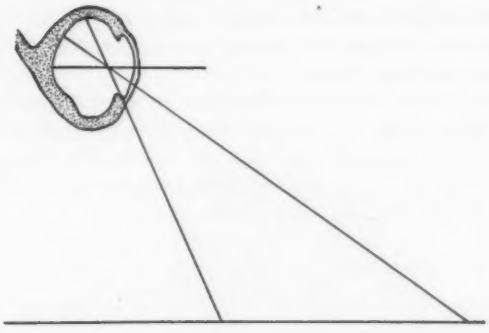
FIG. 2. Sizes of images in upper region of eye when fish was over circles with various diameters. Dorsal ventral diameter of eye—4 mm.; width of head—8 mm.; distance between eye and background—6 mm.; *B.*, background; 3, 5, 7, boundaries of 3-, 5-, and 7-cm. circles.

Paling, therefore, depends greatly upon the size of the white area below the fish.

In previous experiments (Butcher, 1938) where blinders were used in covering the eyes, there was some evidence that illumination from above tended to inhibit the paling response or that paling was more easily elicited when the lower region of the eye was not simultaneously illuminated. There is no way of confirming this observation with a white background below and illumination from above because variation in the illumination from above causes a proportional variation in the reflected light from below. Likewise, if a white bottom is illuminated from below, causing the fish to pale, then illumination cannot be added from above in any way so that paling will not persist.

Whether or not light from above was inhibitory to the paling response was investigated in the following way. A circle of white paper

which would induce paling when placed below a fish and illuminated from above (Fig. 1) was cut into halves. The fish in the glass tube was then placed over half of this circle in such a way that the axis of the fish corresponded with the diameter of the circle (Fig. 5). Fishes arranged in this manner failed to pale, because illumination of the



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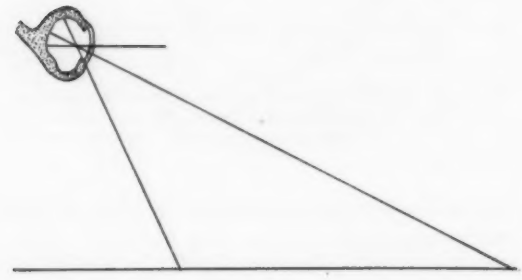

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FIGS. 3 AND 4. These figures illustrate that more of the 3 cm. circle is seen by the small fish than by the large fish. The image in the small fish covers approximately 15 per cent more of the upper region of the retina.

FIG. 3. Eye of fish 80 mm. long, and 10 mm. wide at eye level.

FIG. 4. Eye of fish 40 mm. long, and 6 mm. wide at eye level.

lower regions of both eyes was enough to inhibit any response elicited by the reflected light to the upper region of one eye. Even if the diameter of the circle was greatly increased, paling was not induced in most instances. When the eye which was not over the white semicircle was enucleated, the fish immediately paled, for now the inhibition

resulting from illuminating the lower region of one eye was not enough to prevent the influence of the upper region of one eye. These experiments definitely showed that the paling response was more easily elicited when the lower regions of the eyes were not so intensely illuminated.

The Conditions Necessary for Inducing the Darkening Response

To determine the intensity of illumination of the eye necessary for inducing complete darkening of the body, fishes were placed in glass dishes lined with black paper. These dishes were about 20 cm. in diameter, 8 cm. deep, and contained water 4 cm. deep. A cylinder

FIG. 5. This figure shows how a fish was placed over a semi-circle so that the upper region of only one eye was illuminated by reflected light from below.

lined with a light-proof, black paper enclosed the dishes. The top of the cylinder was covered with opal glass, and a Mazda lamp, inside-frosted, was suspended above the cylinder as the source of illumination. The diffusing opal glass was 18 cm. from the surface of the water in the dish. Two small openings were made in the side of the cylinder. One was used for observing the fish and the other was large enough for transferring fish in and out of the black dish. The temperature of the water was kept at about 16° C. It was always ascertained if fish would assume both pale and dark shades before they were used for the experiments.

In investigating the effect of illuminating the lower region of the retina with different intensities three or four fishes were placed in the dish and allowed to remain for 20 minutes. Meanwhile, control

fishes in other black dishes outside of the cylinder were being exposed to intensities from Mazda lamps which definitely induced maximum darkness. At the termination of 20 minutes, one studied the experimental fishes through the small hole in the side of the cylinder and observed their shades. In order to determine more definitely how many were completely dark and the correctness of the observations made in the experimental dish, the observer then viewed the control fishes, and without changing his field of vision, he quickly transferred an experimental animal into the control dish. This method involved only a few seconds and reduced the possibilities of error as much as any method used. Fishes were tested only once and then discarded.

When the intensity of illumination reaching the fishes was reduced to 2 footcandles, the majority of them failed to completely darken. Exposing 60 fishes to this intensity, 40 per cent of them became completely dark, 20 per cent darkish, and 40 per cent intermediate. The tables in the article by Brown (1936) show that an intensity of illumination of 1.75 footcandles caused complete dispersion of melanin in many *Ericymba buccata* Cope, the silver-mouthed minnow. Danielson (1938) reports that complete melanophore change appeared to occur at and above 1 footcandle in *Nocomis biguttatus* Kirtland. There are undoubtedly variations between different species and variations in threshold between different individuals. When the intensity was increased to 3.5 footcandles and 54 fish were tested, 45 became completely dark, and the other 9 had a darkish appearance.

Since it was necessary to stimulate a large area of receptors in the upper region of the retina in order to induce paling of the body, a few investigations were undertaken to determine the size of the source of light to the lower region necessary to cause the darkening response. In place of the opal glass covering of the cylinder, a black lid was substituted. This covering contained a central aperture, the size of which could be varied. When this aperture was 1.5 cm. in diameter and the fish were receiving 2 footcandles, about the same percentage (40 per cent) became maximally dark as when they received 2 footcandles through opal glass. Only 25 per cent, however, definitely assumed a maximum darkness when the diameter of the aperture was reduced to 1 cm. and they received an intensity of 2 footcandles.

Some *Funduli* thus become maximally dark when receiving an intensity of 2 footcandles from a source of light 1 cm. in diameter. The diameter of the image formed by a source of light 1 cm. in diameter and 18 cm. from the eye is only about .085 mm. or 85 micra. Whether or not more than 25 per cent of the fish will be induced to darken when receiving an intensity greater than 2 footcandles from a source 1 cm.

in diameter has not been determined. At least, a much smaller image induces darkening than the image necessary to elicit paling of the body. There are also undoubtedly individual differences in threshold.

Evidence that the Shade of the Fish Depends upon the Ratio between the Light from Above and the Light from Below Entering the Eye

Observations made by Sumner (1911), and Mast (1916) show that the shade of the flounder's body does not depend upon a visual comparison between its body surface and the background. It seems more probable from their experiments and those of Sumner and Keys (1929) that the ratio between the light coming from above and that reflected from below supplies the stimulus to the eye which enables the fish to assume a certain shade.

To learn if the ratio of light was responsible for the shade assumed by *Fundulus* it was first necessary to secure backgrounds which ranged in shade from white to black, to determine the response of the fish with each background, and the ratio of the direct to the reflected light in each instance. Various gray papers were used for these backgrounds and these were placed in the bottoms of large crystallizing dishes, the sides of which were lined with black paper. These crystallizing dishes were held with clamps about two feet from a table in a dark room. For illuminating the bottom of the dish, Mazda inside-frosted lamps were placed both above and below the dishes.

The response of the fish, the kind of bottom, and the brightness of the bottom when illuminated only from above are recorded in Table II. The responses of 15 fish were usually determined in each of these experiments. Gray 1, gray 2, and gray 3 were very close to neutral 6, 5, and 3, respectively, of the Munsell "Book of Color." [3]

The higher intensities induced about half of the fish in the dish with gray bottom 2 to pale. Reducing the brightness of the bottom to .1 footlambert when the intensity from above was .5 footcandle caused only a few to pale (Table II). Apparently the percentage of reflected light has to be greater than it is from bottom 2 (50 : 10) to induce all to pale.

If the fish assumes the shade of the background below because of the ratio of the light from above to the light from below entering the eye, then fish should pale over gray 3 when its brightness is increased by illumination from below, and a ratio is established which is known to induce paling of the body. To test this hypothesis the Mazda lamp, inside-frosted, was turned on under gray 3 while the intensity of

[3] "The atlas of the Munsell Color System," Munsell Color Company, Inc., Baltimore, Maryland.

illumination from above remained 5 footcandles. When the brightness of this paper was 3 footlamberts or a ratio (5 : 3) existed which induced paling over a white background, the fish, likewise, paled over this gray 3. With added illumination (400 footcandles) from above so that the ratio was 50 : 3.3, an intermediate shade was quickly assumed.

If either gray 1, 2, or 3 were illuminated only from below and their brightness was 1 footlambert (ratio in this instance is 0 : 1), all fish quickly paled. With no illumination from above fish could undoubtedly be induced to pale when the brightness of the bottom was much less than 1 footlambert for they have paled when the background was .2 footlambert in brightness and the intensity from above was .5 footcandle (Table II).

TABLE II

Light relations and shade of fish's body with different shades of paper below fish. *P.*, pale; *SP.*, slightly pale; *Int.*, intermediate.

Shade of paper below fish (sides of container black)	Intensity of light from above footcandles	Brightness of background below fish footlamberts	Ratio of light from above to brightness of background below	Shade assumed by fish
White	400	220	50 : 27	P
	10	5.5	50 : 27	P
	5	2.75	50 : 27	P
	.5	.275	50 : 27	P
Gray 1	400	160	50 : 20	P
	10	4	50 : 20	P
	5	2	50 : 20	P
	.5	.2	50 : 20	P
Gray 2	400	80	50 : 10	50% P, 50% SP
	10	2	50 : 10	50% P, 50% SP
	5	1	50 : 10	50% P, 50% SP
	.5	.1	50 : 10	20% P, 80% SP
Gray 3	400	24	50 : 3	Int.
	10	.6	50 : 3	Int.
	5	.3	50 : 3	Int.
	.5	.03	50 : 3	Int.

Some fish, therefore, paled when the ratio of direct to reflected light was 50 : 10. Fish failed to pale over a gray background below when lighted from above because this background did not reflect enough light in comparison to the light coming from above. If the gray background were illuminated from below so that its brightness was increased, then the fish paled.

Summary

Only a small area of the lower region of the eye of *Fundulus* needs be illuminated to induce a darkening of the body, since as little light as 2 footcandles coming from a source 1 cm. in diameter and 18 cm. above the fish elicits the darkening response. Darkening cannot be induced by illuminating the lower region when there is much illumination to the upper region of the eye.

For eliciting paling of the body, a large area of the upper region of the eye must be illuminated. This is shown by experiments with fish over circles. Regardless of the brightness of a circle 3 cm. in diameter beneath the fish, those 50 mm. in length failed to pale. When the illuminated circle was increased in size, fish paled readily. Paling, therefore, depends greatly upon the size of the white area seen by the fish. A Mazda lamp arranged below a fish so as to illuminate the upper region of the eye thus fails to induce paling because its image does not fall upon enough retinal receptors. Paling is more easily elicited when the lower region of the eye is not illuminated at the same time that the upper region is illuminated.

The ratio between the direct and the reflected light, known to exist with a white background below the fish, has been created with gray bottoms by illuminating them both from above and below. Gray backgrounds illuminated in this way have caused fish to pale readily. The shade assumed by *Funduli*, therefore, depends upon the ratio between the direct and the reflected light entering the eye.

As the percentage of reflected light to the upper region of the eye is increased, there is induced a proportional increase in the inhibitory reaction which causes a reduction in the degree of darkening of the fish.

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OBSERVATIONS UPON AMPHIBIAN DEUTOPLASM AND ITS RELATION TO EMBRYONIC AND EARLY LARVAL DEVELOPMENT [1]

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During early ontogeny, several distinct morphogenic processes proceed more or less synchronously whereas others tend to alternate (Richards, 1935). In the exponential period, described by Schmalhausen (1930), mitotic activity dominates; but with the onset of gastrulation, the mitotic rate falls in close correlation with an increase in differentiation (initiation of the parabolic period). It is at this time, just as the primary caudo-cephalic axis is about to be laid down, that the first embryonic organizers become evident in the dorsal blastoporal lip (at least in Amphibia) and also that important mitotic centers are set up which feed cells into specific regions where they later differentiate into various anlagen, in some cases, at least, under the influence of induction (Derrick, 1937; Self, 1937; Bragg, 1938; Jones, 1939). Behind these more or less morphological manifestations are the actions of the genes, inductors, possibly hormones, etc. which, working through the visible morphological configurations of the cells or their parts, actually are the basic underlying factors in the production of the embryo, and hence of the adult body.

From these considerations, it is evident that the basic factors in embryonic development are essentially physiological, rather than morphological, in character. Studies of cell-migrations or of morphogenic movements (Vogt, 1929; Wetzel, 1929; Gräper, 1929; Pasteels, 1936; etc.), or mitotic indices (Minot, 1908; Self, 1937; Derrick, 1937; Bragg, 1938; Jones, 1939, etc.), and all similar attacks upon the problem of embryological organization cannot, each method of itself, explain morphogenesis. Such studies are valuable mostly as indicating changes in the morphological configurations of parts which in turn are indirect evidences of the basic physico-chemical changes in the protoplasm, a detailed understanding of which can only be attained by physiological methods. Sometime in the future, therefore, we may expect a synthesis of the observations made by the various methods now in use wherein the relationship between cell division, and

[1] Contribution from the Zoölogical Laboratory of the University of Oklahoma, No. 199.

mitotic centers, induction, cell-migration, increase in size, histological differentiation, problems of cell-size and body-size, nucleo-cytoplasmic ratios, the mode of genic action, etc. will all be correlated into one basic biological principle, only fragmentary glimpses of which any one of us now sees by the results of his own special method.

It is well established that the yolky materials in an egg of an animal constitute reserve food which is utilized during some phase of ontogeny as a source of energy, of building materials, or of both: but at just what phase of development and for what processes they are utilized by the animal has apparently received but slight attention (see, however, Saint-Halaire, 1914). During a recent study of the relation of cell division to early embryonic organization of a toad (Bragg, 1938), it was observed that the yolk granules maintained their initial sizes, shapes, and appearances at least to the stage in which the neural tube closed. From indirect evidence, it was also strongly suspected that the embryo did not increase materially in protoplasmic mass up to this stage of development. If these two conclusions were substantially correct, this could only mean that the yolk was not used during the exponential period nor even during the earlier portion of the parabolic period wherein all of the anlagen of the major organ systems were laid down. In other words, the yolk contributed neither energy for the very actively katabolic process of cleavage nor materials for the increase in the size of the body up to this stage of development in the embryos of the species investigated.

Since these observations were somewhat incidental to the main subject of the former paper, and, further, since the yolk must bear important relationships to some of the ontogenetic processes indicated above, it seemed wise to study the yolk in greater detail in order to establish when and where its utilization begins and, so far as possible by the methods used, for what embryological processes it is utilized. It is also of interest to ascertain whether the species used in the former study (*Bufo cognatus*) is peculiar in these matters or whether other amphibian species manifest the same phenomena.

Materials and Methods

The embryos used were those of *Bufo cognatus* Say, *B. woodhousii woodhousii* (Girard), *Rana sphenocephala* (Cope), and *Scaphiopus hammondii* Baird, all from the vicinity of Norman, Oklahoma.[2] Pre-

[2] I am indebted to the following for the use of slides of embryos and larvae prepared and owned by them: to Mr. Virgil Johnson for all stages of *B. w. woodhousii;* to Dr. Minnie S. Trowbridge for embryos of *Scaphiopus;* and to Mr. Robert Taylor for larvae of *Scaphiopus.*

The species of *Scaphiopus* used is the same as that called tentatively *S. bombifrons* Cope by Trowbridge and Trowbridge (1937). In a forthcoming paper, Dr.

pared slides of early cleavage, of the blastula, and of the gastrula of the California newt, *Triturus torosus*, were studied also for comparison with the anuran embryos.

The methods were those commonly employed for embryological work. Embryos and larvae were fixed in one of several different fixing fluids (Smith's, Goldsmith's, and Bouin's, most commonly), dehydrated with ethyl alcohol, embedded by the method of Hamlett, and serially sectioned (6–12 micra). Heidenhain's haematoxylin, alum haematoxylin, and alum cochineal were the principal stains used. The exact procedure made little difference for the purpose of the study. Observations upon living embryos and larvae of all species used except the newt were also made.

Following the same method as earlier (Bragg, 1938), the yolk granules in selected regions were drawn under oil-immersion lenses by means of a camera lucida, all carefully to the same scale. The pictures so obtained were then compared with each other and with the details of structure as seen in the microscopic fields. The facts

Minnie S. Trowbridge and the author will show that the species name, *bombifrons* is not a synonym for *hammondii* as assumed by Wright and Wright (1933) and that the species in question here is *S. hammondii*.

EXPLANATION OF FIGURES

All figures in the plates drawn by camera lucida and to the same scale in order that they may be compared with one another directly. All are of complexes of yolk granules characteristic of the region given for each except Figs. 44 to 49.

A. Blastula No. 12A1, comparable to Bragg, 1938, Stage *A*.
FIG. 1. Micromere.
FIG. 2. Intermediate zone.
FIG. 3. Macromere.

B. Gastrula No. 263A2, comparable to Stage *C* (Bragg, 1938).
FIG. 4. Dorsal ectoderm.
FIG. 5. Just inside the dorsal lip of the blastopore.
FIG. 6. Anterior ectoderm (opposite the yolk plug).
FIG. 7. Dorsal blastopore region. The blastoporal groove between a cell of the dorsal lip (left) and a cell of the yolk plug.
FIG. 8. Condition a short distance inside the blastopore at the dorsal lip. Condition of yolk intermediate between those shown in Figs. 5 and 10.
FIG. 9. Ventral lip of the blastopore.
FIG. 10. Inner yolk mass.

C. Stage of the crescentic blastopore, No. 54A4, comparable to Stage *B* (Bragg, 1938).
FIG. 11. Micromere near the animal pole.
FIG. 12. Innermost yolk cells.
FIG. 13. Region of the dorsal blastoporal groove; compare with Fig. 7.

D. Neural plate stage. No. 75XA1, comparable to Stage *D* (Bragg, 1938).
FIG. 14. Neural plate.
FIG. 15. Dorsal endoderm.
FIG. 16. Lateral mesoderm.
FIG. 17. Lateral ectoderm.
FIG. 18. Ventral ectoderm.
FIG. 19. Ventral yolk mass.

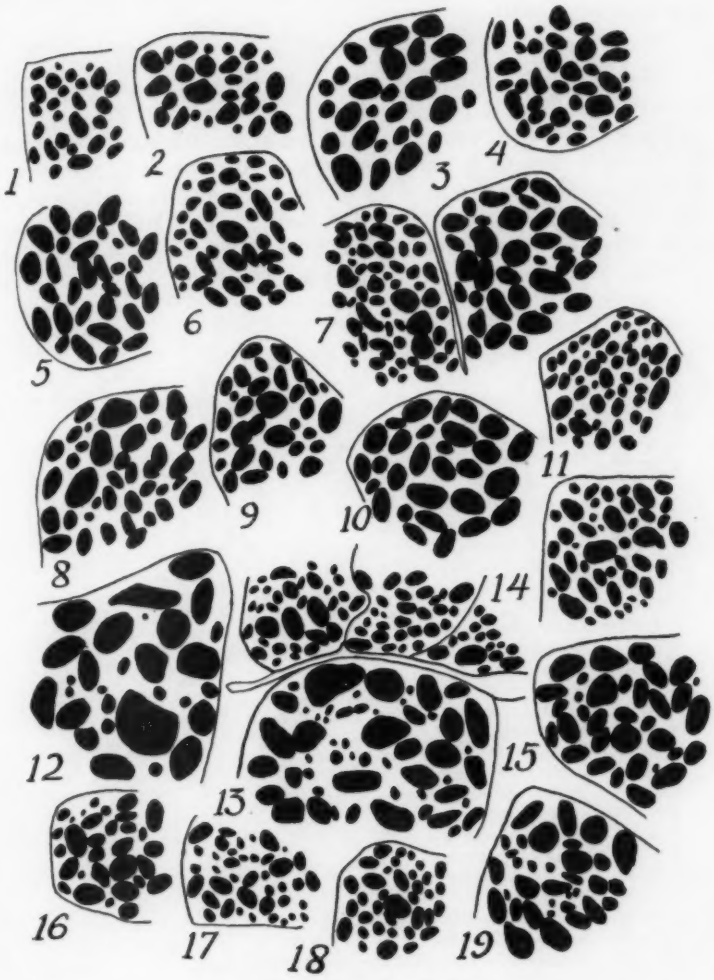

PLATE I. *Bufo cognatus*

gathered in this manner were then correlated with the known stage of development of the individual animals from which the slides had originally been made.

Observations

The distribution and sizes of the yolk granules of all species used followed the general pattern already described for *Bufo cognatus* (Bragg, 1938). Briefly, a gradient of size exists, the smallest granules being mostly located in the animal region, the largest in the vegetal portion of the egg. Species differ in the absolute sizes of the granules but the mode of distribution is the same in all. During cleavage, three types of blastomeres become recognizable, each easily differentiated from the others by the type of yolk granules contained.

Explanation of Figures—Plate II

Fig. 20. Dorsal mesoderm.
Fig. 21. Notochord.
E. Neural tube not quite closed. No. 112A3, comparable to Stage *E* (Bragg, 1938).
Fig. 22. Dorsal mesoderm.
Fig. 23. Superficial lateral ectoderm.
Fig. 24. Neural tube.
Fig. 25. Lateral endoderm.
Fig. 26. Endo-chordo-mesoderm.
Fig. 27. Lateral mesoderm.
Fig. 28. Dorsal endoderm.
Fig. 29. Notochord.
F. Neurula. No. 273a.
Fig. 30. Optical vesicle.
Fig. 31. Ectoderm adjacent to the adhesive organ.
Fig. 32. Brain.
Fig. 33. Adhesive organ.
Fig. 34. Ventral ectoderm.
Fig. 35. Notochord.
Fig. 36. Mesenchyme of the head.
Fig. 37. Lateral mesoderm.
Fig. 38. Ventral yolk mass.
Fig. 39. Lateral ectoderm.
Fig. 40. Dorsal endoderm.
Fig. 41. Somite mesoderm.
Fig. 42. Ectoderm of the head.
Fig. 43. Nerve cord.
G. Larva of 3 mm. total length. No. 134 2-2.
Fig. 44. Ventral yolk mass.
Fig. 45. Mesoderm. Note that the yolk is being used.
Fig. 46. Superficial ectoderm. Yolk nearly gone.
H. Larva of 5 mm. total length. No.141E1.
Fig. 47. Myomere of the tail.
Fig. 48. Section of the nerve cord dorsal to the yolk region (Fig. 44). Yolk granules scattered and small.
Fig. 49. Outline of a fold of superficial ectoderm with only the yolk granules shown.

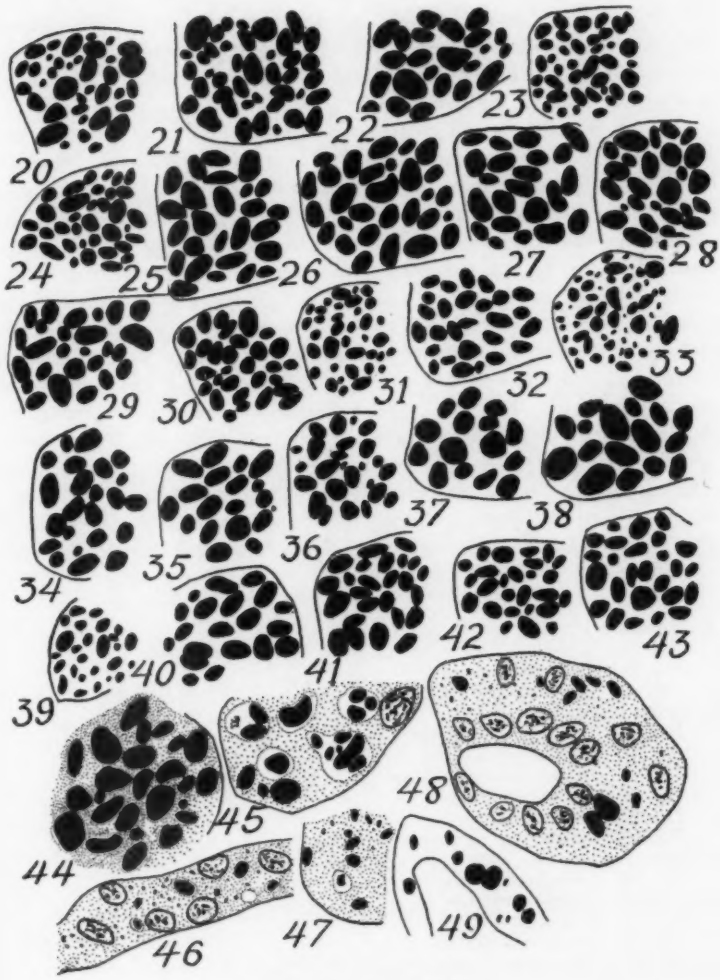

PLATE II. *Bufo cognatus*

These are (1) the micromeres which contain mostly small granules, (2) the macromeres which contain mostly large granules, and (3) cells located in a thick section between these which contain granules mixed and intermediate as to size. This last-mentioned region has been designated as the intermediate zone. These matters are illustrated in Figs. 1–3, 50–52, and 67–68.

I wish to call particular attention to the fact that it is not the absolute sizes of the yolk granules that is to be emphasized. Rather, it is the general appearance of the complex of granules in each type of cell. Some micromeres contain large granules intermingled with the smaller ones and the converse is true in the macromeres. The appearance is due in part to a greater number of the one type of granules or the other in any given cell and in part to the average sizes of the granules. The average length of the granules from the regions of the embryo of *Bufo cognatus* shown in Figs. 1, 2, 3, and the two portions of Fig. 7, for example, bear approximately the following relationships to one another: 1.0 : 1.2 : 1.6 : 1.1 : 2.2, the last two figures being for the dorsal lip of the blastopore and the adjacent yolk plug, respectively.

During gastrulation, the yolk granules maintain their original relationships as to size and appearance within each type of cell (Figs. 4–15). A striking contrast between the appearance of the complex of yolk granules in the dorsal lip of the blastopore and that in the cells

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EXPLANATION OF FIGURES—PLATE III

A. Blastula No. 1.2a.
- FIG. 50. Micromere.
- FIG. 51. Intermediate zone.
- FIG. 52. Macromere.

B. Neural plate stage.
- FIG. 53. Dorso-lateral endoderm.
- FIG. 54. Ventral yolk mass.
- FIG. 55. Dorso-lateral mesoderm.
- FIG. 56. Ventral ectoderm.
- FIG. 57. Neural plate.
- FIG. 58. Notochord.

C. Open neural groove.
- FIG. 59. Notochord.
- FIG. 60. Lateral ectoderm.
- FIG. 61. Neural fold.
- FIG. 62. Somite mesoderm.
- FIG. 63. Dorso-lateral endoderm.
- FIG. 64. Lateral mesoderm.
- FIG. 65. Ventral yolk mass.

D. Embryo just younger than that from which Figs. 59–65 were taken.
- FIG. 66. Posterior ventral yolk mass.

E. *Triturus torosus*, two-celled stage.
- FIG. 67. Yolk complex near the animal pole.
- FIG. 68. Yolk complex near the vegetal pole.

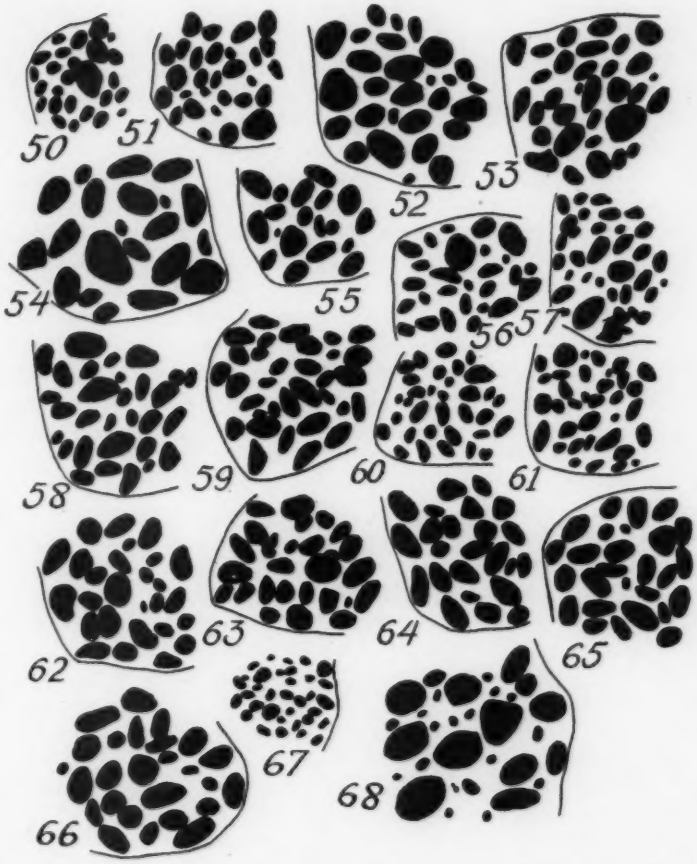


PLATE III. *Bufo woodhousii woodhousii* (except Figs. 67 and 68 which are of *Triturus*).

of the yolk plug just across the blastoporal groove illustrates this fact. (See Figs. 7 and 13.)

In later stages, the essentials in all of the species studied are also as earlier found for *Bufo cognatus*. The micromeres of the blastula pass under the dorsal lip of the blastopore for a short distance only, just enough to cover the yolk plug as the blastopore closes. The micromeres, therefore, give rise mostly only to ectoderm; mesodermal and endodermal derivatives contain only complexes of yolk granules

characteristic of the macromeres of the blastula, except, possibly, some characteristic of the intermediate zone. (See Figs. 14–43 and 59–66.) Hence, it may be concluded without question that in *Bufo*, *Rana*, and *Scaphiopus* and probably also in *Triturus* (stages later than gastrulae not studied) the micromeres differentiate into ectoderm and the remainder of the embryo is derived from the macromeres and the cells of the intermediate zone. Since two orders, four families, and five species appear to agree so closely, it seems very probable that the principles here discussed will be found to apply generally to Amphibia.

In order to determine whether the embryo increases in size during early development, the measurements of embryos and larvae summarized in Table I were made. Taking change in diameter for stages through gastrulation and length thereafter as a measurement of growth, the figures show an increase between the early cleavage stages and the neural plate stage of 33 per cent and a width increase of 16 per cent. At the neural tube stage, the increase in length is 1 per cent more but the width has decreased 16 per cent. Between the neurula and the stage at hatching, the increase in length has reached 136.8 per cent of the diameter at early cleavage and the outer configurations of the embryo have become so irregular that exact measurements of width at any one level of the body-axis can have little meaning. A summary of these facts is presented in graphic form in Text-fig. 1.

It is well known that amphibian embryos absorb water during cleavage. Morgan (1906) found an increase in diameter of about 25 per cent between early cleavage and gastrulation in embryonic frogs, about one-half of which was due to the development of the blastocoel. The figure for *Bufo cognatus* is somewhat less than this (18.9 per cent), probably due to interspecific differences. However this may be, the increase in diameter is not too great to be accounted for almost or quite entirely by the absorption of water during cleavage, particularly if one consider the space occupied by the blastocoel. The data, therefore, confirm the earlier conclusion that no increase in protoplasmic mass occurs up to this stage, although the embryo does actually increase in size. Measurements of living embryos of *Rana sphenocephala* substantiate this general result.

In later stages, but prior to hatching, growth in length is quite rapid but the increase in width is not comparable. Cavities (archenteron, neurocoel, etc.) develop which take up space and the cells become progressively smaller, particularly in areas of high mitotic rate (Bragg, 1938; 1939). While by no means demonstrated, it seems very probable from these considerations that most of the increase in bulk prior to hatching takes place without material increase in funda-

mental protoplasmic constituents (except, of course, the ever present water). This conclusion, moreover, is strengthened by a study of the

TABLE I

Bufo cognatus. Growth of embryos and larvae as measured by length and width after preservation in 70 per cent alcohol. Measurements in mm. to the nearest 0.01. Animals grown in the laboratory at room temperatures (approximately 18–22° C.); cultures maintained in tap water with algae added as food after hatching.

Stage	Age (hrs.)	No. used	Length	Width	Increase between stages		Total increase		Percentage of total increase	
					Length	Width	Length	Width	Length	Width
Early cleavage (2–8 cells)	1–2	20	1.06	1.06	—	—	—	—	—	—
Mid-cleavage	3–6	20	1.12	1.12	0.06	0.06	0.06	0.06	5.6+	5.6+
Gastrulation	18–20	20	1.26	1.26	0.14	0.14	0.20	0.20	18.9−	18.9−
Neural plate stage	33–35	20	1.41	1.24	0.15	−0.02	0.35	0.18	33.0+	16.0+
Neural tube stage	42–46	20	1.42	0.88	0.01	−0.36	0.36	−0.18	34.0−	−16.0+
Hatching	51–55	91	2.51	—	1.09	—	1.45	—	136.8−	—
Mouth a shallow pit	70–74	39	3.00	—	0.49	—	1.94	—	181.5+	—
Mouth a deep pit	74–100	20	3.15	—	0.15	—	2.09	—	197.2−	—
Mouth first functional	102–106	52	4.91	—	1.76	—	3.85	—	363.2+	—
Ready for metamorphosis	45 (days)	22	25.79	—	20.38	—	24.23	—	2285.8+	—

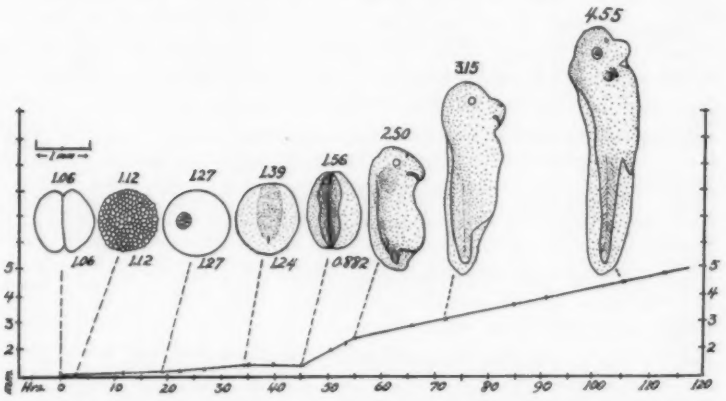
yolk granules in most regions of the embryo, the exceptions occurring during late embryonic life in those areas most active in differentiation.

This is particularly true of *Scaphiopus:* for example, in the adhesive organ of this organism, the yolk granules of a late embryo are noticeably smaller than those in cells from which the anlage of the organ was derived. The same is true of the optic cup. But in the superficial ectoderm of the head and in the brain of the same embryo, they remain essentially unchanged.

From the foregoing observations, therefore, the following general conclusions may be stated:

(1) The embryo increases in size during all phases of development.

(2) This increase does not take place at a constant rate till hatching, after which it does so (at least as measured by length).



TEXT FIG. 1. Graph, increase in average length plotted against age in hours. The figures above the graph are camera lucida drawings, all to the same scale, of representative embryos and larvae of the stages indicated. The numbers above the drawings are the lengths (mm.) of the examples drawn; the numbers below are the widths of these same embryos.

(3) The increase up to the gastrula is due very largely, if not wholly, to the absorption of water, correlated with the space occupied by the development of the blastocoel.

(4) The development of the neurula from the neural plate stage is accomplished with little or no increase in bulk (length increases but width decreases).

(5) The most rapid growth in length occurs between the neurula and the stage at hatching; since the yolk is not altered within most of the cells during the greater part of this period, however, protoplasmic substance is increased but little in the embryo as a whole, even though the bulk of the embryo may increase due to the further absorption of water.

If the yolk is not utilized during early ontogeny, when and for what is it used? Observations upon late embryos and upon early to half-developed larvae of *Bufo cognatus* and *Scaphiopus* indicate the following: (1) The yolk granules begin to break up and to disappear in some regions before they do so in others. (2) Their digestion begins, in general, earliest in those embryonic regions which are first in histological differentiation. (3) Just prior to and during active digestion, vacuoles often appear in the cytoplasm and the yolk granules come to lie in these as though the vacuoles were formed around them: from this it is thought probable that the yolk is digested in intracellular vacuoles into which digestive enzymes pass from the cytoplasm much as in a protozoön.

Figures 30–49 illustrate these processes in *Bufo cognatus*. A cell of the optic vesicle in the neurula contains yolk granules comparable to those in the blastular micromeres (compare Fig. 1 with Fig. 30). In the ventral ectoderm of the same embryo (Fig. 31) they are smaller. In the adhesive organ (Fig. 33) they are not only smaller but also somewhat irregular in shape. The lateral ectoderm contains some irregularly shaped granules but the complexes of yolk in the mesoderm, endoderm, brain, and notochord remain unchanged. (See Figs. 32 and 35–38.) From this it appears that the yolk is used first in ectodermal structures, particularly those in the region of the anlage of the adhesive organ which is soon to differentiate and to function at hatching (Bragg, 1939*a*). In larval stages (Figs. 44–49) the yolk is disappearing in all regions except the ventral yolk mass.

Embryos of *Rana sphenocephala* show similar trends. In late embryos and early larvae, the yolk is beginning to be utilized in the brain, notochord, and the optic vesicle. The superficial ectoderm is probably just beginning to utilize the yolk but there has been no visible change in the mesodermal and endodermal portions. The more cephalic portions of the anlage of the central nervous system begin the use of the yolk before the more posterior portions. This illustrates the use of the yolk in correlation with anterior and cephalic differentiation in general as opposed to posterior and ventral differentiation.

These conceptions are further illustrated by the study of embryos and larvae of *Scaphiopus*. Little if any yolk is utilized before the neural tube is being formed. However, immediately after the neural tube closes, the differentiation of anterior and dorsal structures is well under way. This is especially noticeable in the adhesive organ but it apparently starts in the mesenchyme of the head before it does in the brain or superficial ectoderm in this species. The yolk granules in the

notochord appear slightly decreased in size but those of the posterior and middle ectoderm, somite mesoderm, ventral yolk mass, and endoderm are still unchanged.

Just before hatching, the relation of the disappearance of the yolk to histological differentiation is still more striking. Ectodermal structures and some parts of the mesoderm are losing yolk but endodermal derivatives, for the most part, are not. The dorsal cephalic ectoderm, the optic cup, and the adhesive organ have lost more of the yolk than most of the other parts.

In a 78-hour larva (approximately nine millimeters in total length), differentiation has already reached a functional state in many organs. Some of the potential blood cells have no yolk granules whereas others have a few enclosed in vacuoles. Many contain small particles of yolk with no visible vacuoles around them and some have granules which are apparently unchanged. The superficial ectoderm in all parts of the body has lost much of its yolk. In one embryo, two cells were observed in this layer each of which contained a large vacuole in which were located small particles which stained like yolk. The endodermal wall of the gut still largely retains its yolk although a few of the granules are within vacuoles. The adjacent mesothelial wall of the splanchopleure has relatively few granules, some still quite large, others small. The myotomes of the tail are functional at this time. Sections of this region show the yolk to be small in amount and scattered. The cells of the ventral yolk mass contain granules of various sizes, but since some are definitely located in vacuoles, digestion of yolk has probably just begun in this region.

Discussion

In an earlier paper (1938) it was noted that the mitotic centers in the embryos of *Bufo cognatus* often do not correspond to the centers of susceptibility described by Bellamy (1919) in the embryonic frog and by Hyman (1921, 1926, 1927) and Rulon (1935) in other vertebrates. It was also noted that if the interpretation by these authors of the gradients of susceptibility as metabolic gradients be accepted, one seems justified in thinking of the regions of greatest susceptibility as regions where anabolic metabolism dominates katabolic metabolism. If this be granted, then it follows that histological differentiation is also dominated by anabolic as contrasted with katabolic processes, a conclusion in accord with the distribution of mitotic centers and with the *Gesetzmässigkeit* of Schmalhausen. The place where yolk first begins to disappear in the embryo (dorsal and cephalic regions, especially where most active differentiation is occurring) gives further

evidence for this view, for some of these are the very regions which were found by others to be most susceptible to a variety of harmful influences; and they also tend to be the areas of lowest mitotic rate in certain stages (see especially Bragg, 1938, Table V, p. 165).

During cleavage, katabolic processes dominate and the respiratory relationships of the embryo require much oxygen (Bragg, 1939*a*). The source of the energy used during this period is still unknown, but the results of the study presented herein show clearly that the yolk is not used for this function (nor, indeed, for anything else) during this time.

At gastrulation, the metabolism of the embryo becomes differential, dominated in some regions by anabolism, in others by katabolism. This is shown both by the distribution of the mitotic indices at this stage and by the fact that a secondary center of susceptibility is set up in the dorsal lip of the blastopore (Bellamy, 1919). Since, however, the mitotic rate drops very suddenly at this stage, the embryo as a whole is likely dominated by constructive metabolism; but this is probably only another way of saying that the embryo enters the parabolic period of Schmalhausen. It seems probable, also, that early induction is anabolic in character, since the organizer of Spemann is located in the dorsal blastoporal lip and thus coincides with a center of susceptibility. This is, of course, only what one might expect, inasmuch as the fundamental function of induction seems to be the stimulation of cells to construct embryonic parts which would not arise, at least at a given time or place, without it. Furthermore, all of this correlates nicely with the distribution of the mitotic centers in the gastrula (Bragg, 1938, Table III, p. 161).

The time between early gastrulation and the formation of the neural tube seems to be one of great reorganization. The size of the embryo increases only insignificantly (Table I, Text Fig. 1) and the yolk remains inert; but in this short period (about twenty-five hours at ordinary temperatures in *Bufo cognatus* and probably even shorter in *Scaphiopus*), bilaterality is established, the notochordal and mesodermal anlagen make their appearance and the fundament of the whole central nervous system is formed. The distribution of the paths of cell-migrations and other morphogenic movements (Vogt, 1929 and others), as well as the places of greatest mitotic activity, seem best interpreted to mean that this reorganization is brought about almost wholly by cell-migration from specific centers of katabolic (mitotic) activity at specific places and times.

Following closure of the neural folds no further data on the mitotic indices in the amphibian embryo are available at the present time, but

one would expect from the work on other forms that the mitotic rate in the embryo as a whole would progressively decline and that centers of high mitotic index would continue to arise, particularly just prior to the formation of specific anlagen (Derrick, 1937; Self, 1937; Jones, 1939). The rate of growth in length is greatly increased during the period between the neurula and hatching (a period of about ten hours in *Bufo cognatus* at room temperatures), but this involves the disappearance of the yolk from the cells only in the later stages (except in *Scaphiopus*). It seems probable, therefore, that the increase in mass is only slight and the apparent growth is due to the space taken up, in part by the development of cavities within the embryo, in part by decrease in width relative to length, and in part by further absorption of water. The yolk seems to be used at a slightly earlier period by *Scaphiopus* than in any of the other forms studied and this may be correlated with the exceptionally high rate of development which this form has (Trowbridge and Trowbridge, 1937; Trowbridge, 1939). However, even in *Scaphiopus* the yolk is used first by the regions of most active differentiation, mostly dorsal and cephalic in the embryo.

Late in embryonic life, the curve of growth becomes a straight line and from this time on the yolk progressively disappears from the cells, being used last in the large yolk mass ventral to the lumen of the gut.

Whether, in larval stages, the yolk is utilized primarily for histological differentiation or for increase in the bulk of the protoplasm could not be ascertained with certainty, since these two anabolic processes occur together. The methods used in this study could not, therefore, distinguish between them so far as their relations to the disappearance of the yolk is concerned.

Summary

Sections of embryos and of larvae of several Amphibia, representing two orders, four families, and five species indicate that the yolk is carried passively in most cells till late in the embryonic period. Just before hatching in most species, but somewhat earlier in *Scaphiopus hammondii*, disappearance of the yolk begins in the areas of earliest histological differentiation, mostly dorsal and cephalic in the embryo. Since the regions of greatest susceptibility to injury reported by others are often the ones of lowest mitotic rate, it seems probable that histological differentiation is dominated by anabolic, rather than by katabolic, processes, just as growth must be. Similar reasoning shows that embryonic induction in the dorsal lip of the blastopore is also predominantly anabolic. This is indicated by the correlation

of the results of four methods of attack; the *Getzmässigkeit* of Schmalhausen, the mitotic index, studies of cell migration, and differential susceptibility to injurious environments, as reported by various workers, both in Europe and in America.

One interesting result for which no explanation is offered is that the yolk is not used during early ontogeny by any of the forms studied. This leaves no explanation for the source of the energy required by the very actively katabolic process of cleavage. Little if any increase in protoplasmic mass occurs before the yolk begins to be utilized. It is still uncertain whether the yolk serves primarily for increase in protoplasmic mass, for histological differentiation, or for both, since these predominantly anabolic processes proceed concurrently in the late embryonic and larval periods. However, since the process of early embryonic organization and the laying down of most of the fundaments of the major organ systems occur before the yolk is used, it is clear that all of the early morphological manifestations (whatever their individual natures) proceed normally without the aid of the yolk.

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FURTHER EXPERIMENTS ON THE DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

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In an earlier paper we reported (1937) that the cycle of decomposition and regeneration of nitrogenous organic matter in sea water can be reproduced experimentally. The main stages in this cycle are: living organism—dead organism—ammonia—nitrite—nitrate—living organism. In view of the importance of nitrogenous material in the economy of the sea, it seemed worth while to carry these experiments somewhat further, and especially to consider the following questions:

1. Is it possible to reproduce more than one cycle in the same water?
2. Can the cycle be made shorter by eliminating certain stages?
3. Do successive cycles differ significantly in character or in rates of development?
4. In what ways do anaerobic and aerobic decomposition differ?
5. How completely can the changes observed in the different forms of nitrogen be accounted for in terms of each other; in other words, how constant is the quantitative balance?

Methods and Material

The plan of the experiments was the same as in our previous investigation. Organic material was suspended in sea water in large carboys and allowed to decompose in the dark, during which time chemical analyses were made periodically. Artificial cultures of *Nitzschia Closterium* were chosen as a source of organic matter, for our previous experience had shown these diatoms to be more satisfactory from an analytical standpoint than mixed plankton. Waksman, Stokes and Butler (1937) also used them successfully for a somewhat similar purpose. The diatoms were separated from the culture medium by centrifugation, washed several times with nitrate-free sea water, and finally suspended in a carboy of sea water which had been filtered through No. 4 sintered-glass. All experiments were carried out at the

[1] Contribution No. 222 from the Woods Hole Oceanographic Institution.

uncontrolled room temperature, varying from 15° C. in the winter to 25° in the summer. Before samples were removed for analysis the carboys were shaken vigorously to distribute the suspended matter evenly. When the latter showed any tendency to stick to the glass it was loosened with a rubber-tipped glass rod before shaking, after the rubber had been carefully cleaned to avoid contamination.

The methods for the determination of particulate nitrogen, ammonia, nitrite and nitrate were the same as those used in our previous experiments and have already been described in detail.

The Possibility of Consecutive Cycles

In our previous investigation we found that the nitrate resulting from plankton decomposition could be regenerated into diatom protoplasm. This raised the question of the possibility of repeating such a cycle of decomposition and regeneration more than once in the same water. When the original plankton has undergone decomposition and the nitrate stage has been reached the water is inoculated with fresh *Nitzschia*. After about a week in the light an abundant growth is always observed. The nitrate drops to a minimum and when placed in the dark this newly-developed plankton undergoes decomposition again. In Series 12 three complete, successive cycles were carried out in this way. In another similar series, for which the data are not given, two cycles were completed, and in several others a part of the second cycle.

A poor diatom growth was observed after the second cycle in Series 12 (Table I), in which unidentified algae developed, among the *Nitzschia*.

It was important to know if a regeneration of phytoplankton material is possible in parts of the decomposition cycle other than the nitrate stage. During the plankton decomposition in Series 23, when the ammonia had reached its maximum, but before nitrite or nitrate had appeared, a portion of the water was transferred to another carboy (23A). After inoculation with *Nitzschia* and a week's exposure to light the ammonia had almost entirely disappeared and a heavy diatom growth had occurred. In a similar way Series 19A was removed from No. 19 before the nitrate had reached its maximum. Here, too, diatoms developed rapidly and abundantly and the soluble nitrogen compounds disappeared almost quantitatively. At these stages, at least, no toxic substances had been formed, or deficiencies developed, which could inhibit the development of phytoplankton. "Short-cuts" in the nitrogen cycle can evidently take place, and the possibility of their occurrence in nature suggests an explanation for

TABLE I

Series 12. Woods Hole harbor water. Fresh culture of *Nitzschia Closterium*. Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count × 10^3 per ml.
7– 5–37	256	31	0	14	14	301	174
7	225	65	0	14	14		134
11	107	215	0				12
17	92		0	19	18		3
23	78		0	11	11		<1
30	49	260	0	11	11	320	<1
8– 5	46		1	11	10		<1
13	45		5	25	19		<1
20	40	210	105	110	5	360	<1
24		70					
27	41	30	265	365	0	435	<1
9– 2	31	13	350	355	5	402	
12			390	395	5		
25		25	370	390	20		
10–28	37	61	0	305	305	413	
31	Reinoculated with diatoms and put in light						
11– 7							137
13							780
16	455	21	0	2	2	478	
16			Put in dark				
21							706
12– 1	164						40
24		59	0				
2– 1–38			1	8	7		
4		247					
24	189	246	1			(440±)	
5– 6		0	70	350	280		
29			0				
6–25	142	12	1	300	300	455	
7– 5	Reinoculated with diatoms and put in light						
12	408			80	80?	(490±)	
15				10			
18	489	0	0	40	40	529	
18			Put in dark			*	
29	304	115	0	25	25	445	
8– 5	190	175	4	18	14	387	
12	202	110	45	50	5	362	
19	196	20	150	155	4	371	
25	208	10	170	160	0	388	
31	237	0	200	(160)	0	437	
9–13	260	18	0	300?	300?	578	

* Dissolved organic nitrogen = 370.

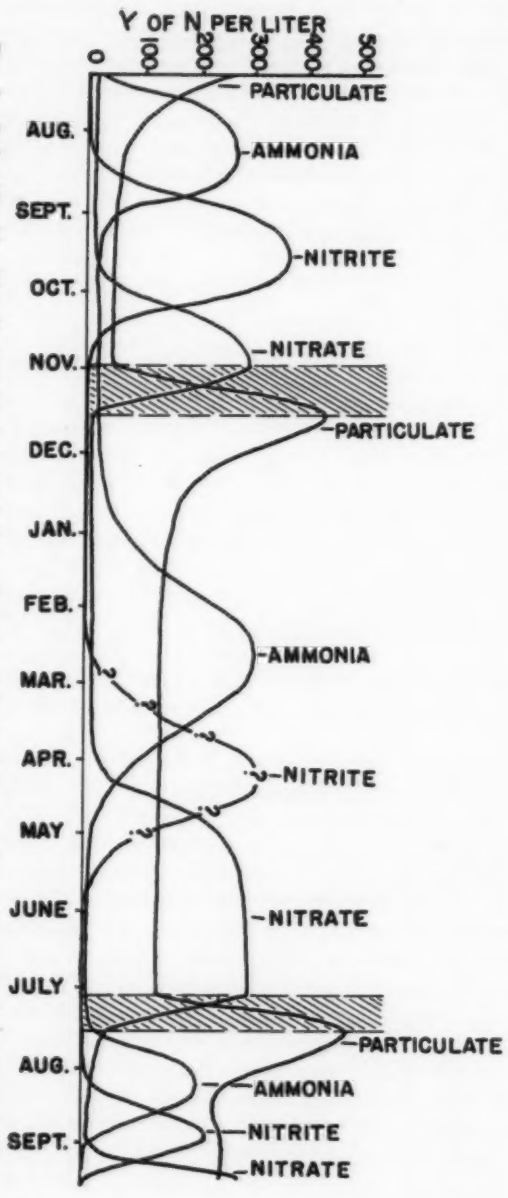


FIG. 1. Series No. 12. The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from Woods Hole harbor. The shaded areas represent regeneration periods, with the culture in the light; the remainder, decomposition in the dark.

the rapid succession of great numbers of phytoplankton organisms belonging to different species. In nature, all stages of the cycle must be taking place simultaneously, and the momentary picture is simply one of equilibrium.

Chemical Observations

Decomposition of Particulate Nitrogen

The particulate nitrogen is that contained not only in diatoms, but also in bacteria and miscellaneous debris. This material began to decompose rapidly in all cases, as soon as the water was placed in the dark. It never disappeared entirely, however, but reached an apparently constant level after periods varying from two to six weeks. This was true not only in the first decomposition cycle but also in the subsequent ones. This residual material is very resistant to further decomposition, and consequently the level of residual particulate nitrogen is higher after each successive decomposition cycle. In the first cycle of Series 12, for example, the residual particulate nitrogen was about 40 γ per liter, in the second cycle about 150 γ, and in the third 200 γ. These amounts are about 16 per cent, 33 per cent and 41 per cent of the particulate nitrogen present at the beginning of each respective cycle. It seems not unlikely that under natural conditions in the sea the plankton is incompletely decomposed, and a large part of the particulate nitrogen found by von Brand (1938) in the deeper levels may be contained in such resistant or slowly decomposing plankton and bacterial residues. The occurrence of bacteria and debris in this resistant fraction is indicated by the fact that the quantity of particulate nitrogen does not consistently follow the diatom count. While the sum of particulate and ammonia nitrogen is fairly constant during the first part of the decomposition cycle, the diatom count falls off much more rapidly than does the particulate nitrogen. Nevertheless, in nature this refractory residue cannot be entirely resistant to decomposition, otherwise the insoluble nitrogenous material, in the water or on the bottom, would increase without limit.

A curious irregularity was observed in Series 25, which was aerated by a constant stream of pure air. After about six weeks of normal decomposition, accompanied by the appearance, first of ammonia and then of nitrite, the particulate nitrogen rose abruptly to its initial value and the ammonia and nitrite disappeared entirely. A microscopic examination, carried out for us by Dr. Lois Lillick, showed the presence of an enormous number of bacteria and a few flagellates. The diatoms had disappeared completely. This phenomenon must be

attributed to the development of a peculiar bacterial flora, since we did not observe it in any other series, including No. 24, which contained the same water and plankton as No. 25 but differed only in not being agitated with an air-stream during the decomposition.

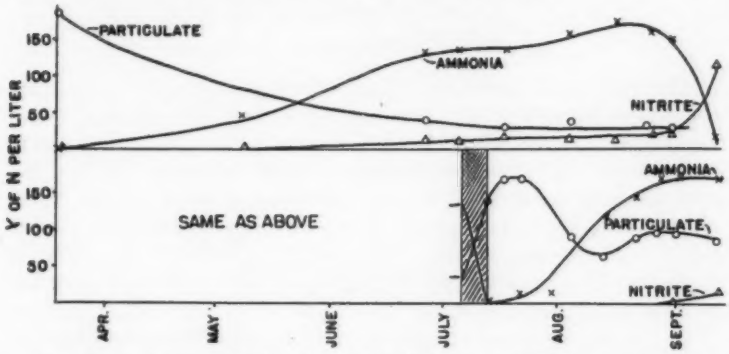
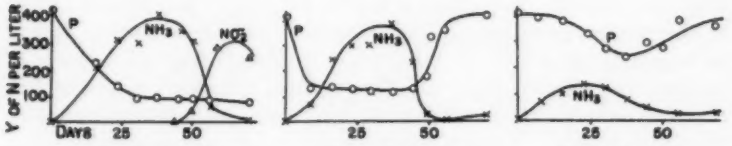


FIG. 2. Series No. 23 (above) and 23A (below). The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, plotted against time. Source of organic matter, fresh culture of *Nitzschia Closterium* suspended in sea water from the Sargasso Sea. The shaded area represents a regeneration period, with the culture in the light; the remainder, decomposition in the dark.



Series No. 24 Series No. 25 Series No. 26

FIG. 3

The disappearance of particulate nitrogen (*P*) and the simultaneous changes in ammonia and nitrite in cultures of *Nitzschia Closterium* in sea water from Woods Hole harbor. The three cultures were identical at the start. Series No. 24 stood quietly in the dark. Through No. 25 a continuous stream of purified air was bubbled. The air was completely removed from Series No. 26 and a continuous stream of purified hydrogen bubbled through it.

Ammonia

During the first decomposition cycle, in all cases, ammonia appeared in the water rapidly and in such amounts as to exclude the possibility of soluble nitrogen compounds intermediate between particulate nitrogen and ammonia. This but confirms our previous findings. But in the second cycle, although the particulate nitrogen disappeared at about the same rate, ammonia was not formed as

rapidly as in the first. In Series 19A, for example, ammonia appeared only after 30 days of decomposition in the dark, during which time the particulate nitrogen had diminished by 160 γ per liter. The same behavior is found in Series 23A and in the second cycle of No. 19, and very likely also in the second cycle of Series 12, although the data are incomplete in the latter. These cases indicate the formation of soluble nitrogen compounds of higher molecular weight, intermediate between dead protoplasm and ammonia. Although there are two or three possible explanations for this lag in ammonia formation, we are not yet inclined to urge the acceptance of any one of them. It is also to be observed that the third cycle of Series 12 resembles the first in its more rapid rate of ammonia appearance.

In the first cycle it generally required from 16 to 25 days for the ammonia to reach its maximum, where it remained for a period of from 21 to 50 days before dropping. A notable exception, however, occurred in Series 22 and 23, in which the ammonia did not appear until about the forty-eighth day, required three to four months to reach its maximum, and remained for another two months before disappearing entirely. This unusually long duration of the ammonia stage would seem to be connected with the source of the water in the experiments. That used in Series 22 came from the Caribbean, and that in Series 23 from the Sargasso Sea, while in all other cases the water was taken from near the shore. Since the diatoms in all experiments were from persistent cultures the bacterial flora introduced with them was presumably constant, but whether the difference in decomposition is due to differences in the bacterial flora of the water itself or to such properties of the water as might influence the growth of bacteria, is not yet clear.

The lag in the oxidation of ammonia to nitrite in Series 22 and 23 was apparently not due to the absence of the necessary bacteria, for portions of these cultures were inoculated with 1 ml. from Series 12, in the midst of its nitrite stage, without any resulting change in the rate of nitrite formation.

Nitrite

In the first cycle nitrite began to appear in 31 to 58 days, corresponding to the beginning of the disappearance of ammonia. It reached its maximum when the ammonia had dropped to a minimum; that is, in a period of from 46 to 74 days. The total duration of the nitrite stage of the first cycle was quite irregular: two months from beginning to end in Series 12, but more than six months in Series 13 (See Table II). The lag in the latter case may in some way be related to the continuous aeration.

Our data concerning nitrite in the second cycle are too scattered to tell whether or not the rate of nitrite formation is the same in the second cycle as in the first. In the third cycle of Series 12 nitrite, like ammonia, developed rapidly.

TABLE II

Series 13. Woods Hole harbor water. Fresh culture *Nitzschia Closterium* in dark, with ammonia-free air bubbling through. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen detd.	Diatom count $\times 10^6$ per ml.
7– 8–37	269	43	1	11	10		234
10	380	11	0	17	17	407	274
13		115	0				
14	280						74
19	109	240	0	11	11	360	2
24	86	300	0	11	11		<1
31	60	310	0	11	11	381	<1
8– 5	53		1	11	10		<1
13	29		0	11	11		<1
20	25	400?	0	9	9	434	<1
24		360					
27	23	330	3	10	7	365	<1
9– 2	34	350	30	35	5	419	
12			330	340	10		
25		15	330	350	20		
10–28	7	82	345	(310)	?		
11–18			321				
2– 1–38		20	272	(260)	?		
24	74	20	232				
5– 6			0	400?	400?		
6–25	120	25	1				
7–15	118	74	7	230	225	422	
7–18		Put in light, not reinoculated					
8– 2	97	20	4	200	200		<1
4	Reinoculated with diatoms						
10	389	9	0	10	10	408	*

* No diatoms present; unidentified algae.

Nitrate

Nitrate begins to appear only when nitrite disappears, and this never seems to happen as long as a significant amount of ammonia remains. Since one cannot rely upon a greater accuracy than 10 per cent in the analytical determination, any quantitative balance is uncertain when large amounts of nitrate are involved.

TABLE III

Series 19. Woods Hole harbor water, collected 2–25–38. Fresh culture *Nitzschia Closterium*. Standing quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10^3 per ml.
2–25–38	316						
26		12	0	10	10	338	275
5– 6		41	12	25	13		
6–25	71	50	87	285	200	406	
7– 6		5	12		(Series 19A separated)		
15	48	15	1	350	350	414	
7–18	Reinoculated with diatoms and put in light						
29				17			
8– 2	396	0	0	10	10	406	56
8– 2	Put in dark						
10	451	0	0	15	15	466	240*
16	262	10	0	20	20	292	220†
23	201	30	0	15	15	246	
30	304?	15	1	20	19	339	
9–13	193	70	0				

Series 19*A*. Portion separated from Series 19 on 7–6–38. Reinoculated with diatoms and put in light.

7– 6		5	12				
12	382	12	0	15	15	409	475
12	Put in dark						
18		0	3	25	22		350
21	396	7	0	10	10	413	
29	252	5	0	15	15	272	5
8– 5	221	90	0	15	15	326	
12	141	175	0	10	10	326	
20	208	186	0	10	10	404	
25	187	250	0	15	15	452	
31	201	250	0	20	20	471	
9–13	171	250	0	10	10	430	

* Few *Nitzschia;* mostly *Skeletonema Costatum.*
† Both *Nitzschia* and *Skeletonema.*

ANAEROBIC DECOMPOSITION

In addition to the experiments already described, which were carried out under aerobic conditions, the anaerobic decomposition of diatoms was also studied in two series. The carboys containing the water and diatoms were first evacuated to remove all the air from the water and the container. Then a slow, continuous stream of purified

hydrogen was bubbled through the water. After decomposition in the dark for some time a strong odor of hydrogen sulfide was observed. In both cases the particulate nitrogen diminished very slowly, but remained constant at a level very much higher than that in the aerobic decompositions. The diatom counts also remained high; in fact, when Series 26 was discontinued, after 10 weeks, living diatoms were still found, which grew when placed in fresh culture medium.

TABLE IV

Series 22. Water from the Caribbean Sea. (18°–35′ N; 79°–14′ W); one year old. Fresh culture *Nitzschia Closterium*. Quiet, in dark. Micrograms of nitrogen per liter.

Date	Particulate	Ammonia	Nitrite	Nitrate + Nitrite	Nitrate	Total nitrogen determined	Diatom count × 10^3 per ml.
3–18–38	123	0	0			125*	213
5– 6		25	0	10	10		
6–25	41	113	12	10	0	166	
7– 6		115	12				
13	22						
15		118	12	25	13		
8– 3	30	150	11	12	1	191	
13		145	12		(Series 22B separated)		
23	23	150	16	20	4	193	
30	30	125	30	30	0	185	
9–13		20	90			(150±)	

Series 22*B*. Portion separated from Series 22 on 8–13–38 and inoculated with 1 ml. from Series 12. In dark.

8–13		145	12				
20		205	22	25	3	160	
30		210	36	35	0	245	
9–13		220	50			(270±)	

* Dissolved organic nitrogen = 93.

Ammonia also increased in this series for the first three weeks, and then gradually disappeared. In the other series no appreciable amount of ammonia was formed; on the contrary, a small amount of that originally present disappeared. This ammonia was not recoverable from the effluent hydrogen, nor are we able as yet to account for the behavior of ammonia in either of these series. As might be expected, no nitrite was formed.

After about two months under anaerobic conditions a portion from one of the cultures was aerated and kept henceforth aerobically.

Two months later a large amount of ammonia had been formed, but no nitrite or nitrate had appeared by the time the experiment was stopped.

Quantitative Balance

In our previous investigation we pointed out that in nearly every case studied the total determined nitrogen in the system (that is, the sum of the particulate nitrogen, ammonia, nitrite and nitrate) increased continuously throughout the period. In the cases we are now reporting the quantitative balance is much more satisfactory. In Series 13, 22, 23, 24 and 25 the changes in total nitrogen are small and probably explainable in terms of accumulated errors. In Series 12, 19, 19A, 23A and one other, the increase observed is too large to be accounted for in this way, but is less, relatively, than the increase noted in our first experiments. Previously, we discussed three possible explanations for this increase in total nitrogen: systematic errors in the determination of particulate nitrogen, the participation of dissolved organic nitrogen in the decomposition, and nitrogen fixation. We were in no position to prefer any one of these explanations. The fact that a good nitrogen balance was observed in half of our later experiments, including one which extended over more than a year, seems definitely to eliminate the possibility of systematic errors. The difficulty of determining dissolved organic nitrogen with any accuracy makes it almost impossible to test the second hypothesis directly. (The one determination given in the data for Series 12 was made, with some difficulty, by the method of Krogh, 1934.) However, we sought to investigate this question indirectly, by trying to see whether ammonia appears, on standing, in water devoid of gross particulate matter, and if so, whether the process is related to the content of dissolved organic matter. Two filtered samples of water, one from Woods Hole harbor and one from the Sargasso Sea, were placed in the dark and the usual determinations made periodically. Previous work had shown that the harbor water had a higher organic nitrogen content than water from the open sea, but there was no difference in the behavior of the two kinds of water. A small increase in nitrogen, about 60 γ per liter, was observed in both. This could indeed be the result of the participation of dissolved organic nitrogen, but we are inclined to believe that it is due to an entirely different cause. All the stored carboys were tightly stoppered, but before the removal of samples they were vigorously shaken and opened. During this time the water comes into contact with a rather considerable quantity of air, from which it may take up ammonia. To test this possibility, clean filtered air was aspirated for 12 hours through a sample of

sterilized Sargasso water. This resulted in an increase of 50 γ of ammonia-N and 20 γ of nitrate-N. Such a quantity of ammonia is not surprising, in view of the amount of decomposing organic matter in this laboratory and its vicinity. It seems quite possible, therefore, that at least a large part of the increase in total nitrogen observed in some of our experiments may be due to contamination from the air. Nevertheless, this is not conclusive, and we are still in no position to exclude the possibility of either nitrogen fixation or participation of dissolved organic nitrogen. Atmospheric contamination was ruled out in Series 13 and 25, through which purified air was aspirated, and in these cases, indeed, the total nitrogen did not increase. Still, no increase was observed in Series 22, 23 and 24, which were not aerated, but which stood side by side with carboys in which nitrogen accumulated. Further work is still being done in the effort to clear up these discrepancies.

Summary

1. Several consecutive cycles of decomposition and regeneration were carried out in the same water.

2. It is confirmed that in the first cycle the main stages of decomposition are: dead body—ammonia—nitrite—nitrate. In the second cycle there is evidence of intermediate soluble substances between dead body and ammonia.

3. Under anaerobic conditions the initial states of decomposition take place more slowly than under aerobic conditions, and no nitrite or nitrate is developed.

4. Regeneration of nitrogen into phytoplankton protoplasm is possible not only in the nitrate stage but also in the ammonia stage and before the nitrate has reached its maximum.

5. The quantitative nitrogen balance was better than that reported in previous experiments, and possible reasons for the discrepancies still present are discussed.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1939

JULY 5

The effect of biologically conditioned medium upon the growth of Colpidium campylum. G. W. Kidder.

The effects of metabolic products upon a population has been the subject of many investigations in the past. Decreased growth rate and population yield due to the accumulated products of metabolism have been reported for many organisms, especially micro-organisms. On the other hand, growth acceleration has been noted in many instances and the effect ascribed to substances given off by like or different species.

Recently we have been interested in making comparisons between some of the phases of protozoan growth and those which have been reported for the bacteria and yeasts. In these studies it has been possible to utilize many of the techniques employed by the bacteriologist due to the fact that the species of protozoa used were all bacteria-free and growing in non-particulate broth. The following results were obtained from studies on the ciliate *Colpidium campylum*.

Manipulation of the conditioned media (2 per cent proteose-peptone-1 per cent dextrose broth) was as follows: *control*—plain, fresh broth; *conditioned*—broth in which *Colpidium* had grown for varying lengths of time and the ciliates centrifuged out; and *filtered conditioned*—conditioned medium which had been passed through a Seitz bacteriological filter. All experiments were performed with the three types of media run in parallel series, at 26° C.

Growth in control flasks (inocula taken from the log phase) exhibited typical logarithmic growth for 28 to 40 hours, depending upon the size of the inoculum. After a very short "negative growth acceleration" period the curve tends to level off and remains at a relatively high level for some weeks. Medium conditioned 60 hours and then inoculated with log ciliates shows a large and significant acceleration during the early growth period. Negative growth acceleration occurs sooner than in the control and the maximum yield is never as great. Filtered medium which has been conditioned 60 hours produces a decided lag phase, indicating inhibition in the early periods of growth. Maximum yield is similar, however, to that in the conditioned (supernatant of a centrifugate). Increasing the length of the conditioning period lowers the curves for both the conditioned and the filtered conditioned media. Slight acceleration in the former was obtained, however, after 4 weeks of conditioning, while the lag period of the latter was increased. Acceleration over the growth of the control was not found after 8 weeks of conditioning although the difference between this curve and that produced in the filtered 8 weeks conditioned was still of the same magnitude as those previously described.

From the results of these experiments it might be said that it appears that substances are released into the medium by growing ciliates which produce a decided effect upon subsequent growth of fresh ciliates. We can think of these substances as falling into two categories, one a growth inhibitor which will pass through an asbestos filter and the other a growth accelerator which will be absorbed on the filter. When both are present together (as in the supernatant of a centrifugate) the accelerator masks the effect of the inhibitor during the early growth phases. When the accelerator has been removed (filtrate) the inhibitor action is marked. There appears to

be a differential as to time of production as the accelerator becomes increasingly less effective as time of conditioning increases while the inhibitor becomes more effective. Until more work is completed upon these very interesting substances the above analysis can only be a conjecture.

Respiration in Chilomonas paramecium. John Hutchens.

The rate of consumption of oxygen and the respiratory quotient of chilomonads grown in sterile, pure, mass cultures in a medium containing CH_3COONa, NH_4Cl, $(NH_4)_2SO_4$, K_2HPO_4, $MgCl_2$, and $CaCl_2$ were measured at 25° C., using simple Warburg respirometers. The rate of consumption of oxygen was found to vary inversely with the age of the culture from which the organisms were taken, and also with the strain of chilomonads used. The respiratory quotient was found to vary directly with the rate of oxygen consumption, *i.e.*, inversely with the age of the culture. The following table compares the rate of oxygen consumption and the respiratory quotient of chilomonads from two different strains at different times following inoculation of the cultures.

The relation between the rate of consumption of oxygen by chilomonads and the respiratory quotient

Age of culture in hours	Strain of chilomonads	Rate of consumption of oxygen in cu. mm. per 10,000 chilomonads	Respiratory quotient
24	2	0.40	0.93
24	1	0.35	0.91
48	2	0.24	0.80
48	2	0.23	0.81
48	2	0.23	0.79
48	1	0.17	—
72	2	0.17	0.75
72	2	0.17	0.74
72	1	0.12	

Temperature and starch and fat in Chilomonas paramecium. Jay A. Smith.

Starch and fat in *Chilomonas* are contained in particles, and by proper staining (Lugol's solution for starch and Sudan III for fat) the size of each particle and the total volume of each substance can be ascertained.

Temperatures from 9.5° C. to 39° C. were used.

The solution, which contains inorganic salts and sodium acetate, was that employed by Mast and Pace and co-workers.

Within viable temperatures, it was found that the volume of starch remains constant, that the volume of fat decreases as the temperature rises, but that the volume of both starch and fat synthesized by the progeny of one chilomonad during a period of 24 hours increases greatly. This indicates that the rate of synthesis within the viable temperature range may be the factor that controls the frequency of division.

At a lethal low temperature (9.5° C.) there is no division, the volume of starch gradually decreases, but the volume of fat increases, which indicates that in the absence of synthesis of starch, starch is transformed into fat.

At lethal high temperatures (32.5°–39° C.) the frequency of division decreases as the temperature rises, and there is an accumulation of starch and fat that reaches a maximum at 35° C. and then decreases. This indicates that the rate of synthesis

of starch and fat increases in the same manner that it did at viable temperatures, and thus the accumulation of starch and fat is due to two interacting factors: the rate of synthesis and the period the chilomonads live.

Thus, the decrease in the frequency of division at lethal high temperatures is probably caused by the same factors that cause death, but there is no relation between the death, the frequency of division *and* the rate of synthesis of starch and fat.

JULY 11

The differentiation of isolated rudiments of the Amblystoma punctatum embryo. Floyd Moser.

Using the technique which has been developed in Harrison's laboratory, a number of structures associated with the embryo of *Amblystoma punctatum* have been isolated and cultured *in vitro* under nearly aseptic conditions. The embryos were generally in Harrison's Stage 29 at the time of operation, though in some cases Stages 27, 28 and 30 were also used. Among the structures isolated were the balancer rudiment, gill rudiment, limb rudiment and tail bud. During the 10 or 11 stages immediately following operation the explants keep pace both in rate and degree of differentiation with the intact structures in unoperated control animals. This, while it is itself of some interest, takes on more special significance in view of the fact that the explants are entirely free of nervous and vascular connections. The tail bud explants alone are exceptions to the latter statement in that they doubtless are well provided with nervous structures.

Typically, the isolation of the ectoderm of the gill rudiment yielded nothing that was gill-like in appearance, while the ectoderm plus the underlying layers of mesoderm gave rise to a single gill. Isolations of gill rudiment consisting of ectoderm, mesectoderm, mesoderm and entoderm gave rise to three gills, which in external appearance, were not unlike those of the unoperated control animals.

Explanted balancer rudiment consisting of ectoderm and the underlying layers of mesoderm and entoderm gave rise to what appeared to be perfect balancers, but these were no better than those obtained from isolated balancer ectoderm alone.

Limb rudiment isolations consisted of the mesoderm and its overlying sheet of ectoderm. As differentiation of the explanted structure takes place, it becomes possible to tell whether the rudiment has come from the right or the left side because of the presence of characteristic surface contours.

When the unoperated controls reach Stages 38 to 39, the isolated tails exhibit function in the sense that they twitch when stimulated by means of slight pressure with a hair-loop.

The present experiments, as well as others involving the fragmentation and fusion of rudiments, and experiments in which isolated rudiments of various ages have been grafted back to host embryos, will be reported at greater length elsewhere.

The production of duplicitas cruciata and multiple heads by regeneration in Planaria. Robert H. Silber.

The observations reported were taken from a paper published in *Physiol. Zool.* (vol. 12, No. 3, July, 1939, p. 285) by Robert H. Silber and Viktor Hamburger and entitled: "The production of duplicitas cruciata and multiple heads by regeneration in Euplanaria tigrina."

Neural differentiation without organizer. L. G. Barth.

Previous experiments have shown that the amphibian ectoderm in the gastrula stage may very easily be stimulated to form a neural plate. This suggested that under certain conditions the ectoderm might form a neural plate without the organizer

or any external stimulus. It was first found that the fusion of two to eight explants of ectoderm would develop with neural tubes, while a single explant formed only a mass of epidermis.

Following this a number of fusions between two pieces were made, some with the antero-posterior axis coincident and some reversed. When the antero-posterior axis of the two explants coincides a neural tube differentiates but when the axes are reversed only epidermis results. Thus the polarity of explants must be preserved in order that a neural tube may form without an organizer.

The neural tube differentiates from the anterior end of the explant since when two explants are united by their anterior ends a neural tube appears in the middle. Further when an anterior half explant is fused with the anterior end of a whole explant the neural tube forms at the end. Other fusions also show that the anterior end forms the neural tubes. There is then an antero-posterior polarity or gradient in the isolated ectoderm and this polarity must be maintained in order to obtain neural tubes without organizer.

This antero-posterior gradient exhibits itself further by differences in oxygen consumption. The roof of the blastocoel was cut into four parts along the antero-posterior axis from the dorsal lip to the ventral epidermis. The results show that the oxygen consumption is high in the anterior pieces of ectoderm and low in posterior pieces. The dorsal lip respires at about the same rate as the anterior end of the ectoderm.

JULY 18

The effect of substrate concentration on the cyanide sensitivity of the oxygen consumption of yeast. Kenneth C. Fisher.

This paper has already appeared in the *Proc. Am. Physiol. Soc.* under the title, "The sensitivity of the oxygen consumption of yeast to cyanide" (*Am. Jour. Physiol.*, **126**, pp. 491–492).

A comparison of cyanide and azide as inhibitors of cell respiration. C. W. J. Armstrong.

This paper was published by C. W. J. Armstrong and Kenneth C. Fisher in the *Proc. Am. Physiol. Soc.* under the title: "The effect of sodium azide on the frequency of the embryonic fish heart" (*Am. Jour. Physiol.*, **126**, p. 423).

The relation of blood to the respiratory ability of fresh water fish. Laurence Irving.

Fresh water which is habitable for fish may be well or poorly oxygenated and may contain CO_2 at practically zero or at a quite noticeable pressure. The properties of the blood for transport of oxygen also vary. Oxygen dissociation curves for the blood of seven common fish at 15° in the absence of CO_2 are similar and differ only in ease of combination with oxygen. The P_{O_2} necessary for half saturation defines the position of the curve for each fish and is as follows: catfish 1.4 mm.; carp 5 mm.; bowfin 4 mm.; common sucker 12 mm.; brown trout 20 mm.; brook trout 22 mm.; rainbow trout 22 mm.

Carbon dioxide increases the P_{O_2} necessary for oxygenation. If the increase in P_{O_2} for half saturation be divided by the P_{CO_2} which effects that change, then the quotient distinguishes the blood of each fish. The quotients are: catfish 0.3; carp 1.0; sucker 3.5; brook, brown and rainbow trout 6.0. The effect of CO_2 on half saturation with oxygen indicates the influence upon unloading of oxygen from blood into the tissues.

Carbon dioxide also reduces the ability of blood to combine with oxygen at 150 mm. pressure of oxygen. The curves relating oxygen contained at $P_{O_2} = 150$

mm. with increasing P_{CO_2} are similar for the various fish and reach limits at $P_{CO_2} = 60$ mm. At these pressures of CO_2 the fraction of the oxygen capacity remaining in the blood is as follows: catfish 1.00; carp 0.90; yellow perch 0.77; sucker 0.58; chain pickerel 0.54; brook trout 0.50; rainbow trout 0.48; brown trout 0.50; lake trout 0.40. The species take the same order on the basis of either ease of saturation with oxygen or magnitude of CO_2 effect.

The effect of CO_2, which must facilitate unloading of oxygen into the tissues, should also hamper oxygenation if CO_2 is present in the water passing over the gills. The ability of the fish to utilize oxygen from water is cut down by CO_2 and in the same order for the species as the order of the CO_2 effect. Trout are unable to utilize oxygen in the presence of much CO_2, but catfish tolerate large pressures of CO_2. While the P_{CO_2} necessary to prevent completely the utilization of oxygen is larger than would occur naturally, the sensitivity of trout to CO_2 is such that some naturally occurring conditions would hamper oxygen utilization and hinder or prevent respiration in such water. In this manner it is shown that the specific properties of blood of fish which facilitate respiratory transport under some conditions would serve as definite barriers to existence of the fish under other conditions.

JULY 25

The relation between fermentation and respiration in higher plants (The Pasteur Effect).[1] David R. Goddard.

The experimental material was thin slices of cortical root tissue of the carrot (*Daucus carota*). All measurements of gaseous exchange were conducted in the Fenn micro-respirometer at 22° C.

It was found that carrot root respiration was strongly inhibited (75–85 per cent) by 10^{-3} M HCN or NaN_3 and about 65 per cent by 95 per cent CO. The CO inhibition was reversed by light. The partition coefficient of the oxidase for CO and O_2 was 9. The results are strong evidence that the major part of the respiration is catalyzed by cytochrome oxidase. Since these same poisons at the above concentration (and 100 per cent CO) did not inhibit fermentation in carrot, these poisons may be used to separate fermentation and respiration in the carrot under aerobic conditions.

Unpoisoned carrot tissue did not show any aerobic fermentation, but the anaerobic fermentation was high. These results demonstrate the existence of the Pasteur effect. As the O_2 pressure is lowered the rate of O_2 consumption begins to fall at about 5 per cent, but fermentation does not occur until $2\frac{1}{2}$ per cent or less. In fact, a very considerable inhibition of respiration (about 45 per cent) may be obtained before fermentation begins. With decreasing respiratory rate, the rate of fermentation increases. Aerobic fermentation may be obtained by poisoning respiration with HCN or NaN_3, but no fermentation occurs until the respiratory inhibition is 45 per cent or greater. In the presence of CO aerobic fermentation also occurs, and this effect may be overcome by light. These experiments indicate that the mechanism of oxygen inhibition of fermentation is through cytochrome oxidase. In the carrot root it has been impossible to poison the Pasteur effect, that is, to obtain aerobic fermentation without respiratory inhibition, with low O_2 tensions, HCN, NaN_3, or ethyl carbylamine. These results are all consistent with Meyerhof's oxidative resynthesis theory as an explanation of the Pasteur effect. They do not prove this theory. The above experiments are consistent with Lipmann's explanation only in the special case that the oxygen oxidation of the fermentation enzymes is catalyzed by cytochrome oxidase.

[1] The results reported here are based on experiments of Mr. Paul Marsh and the author; and a full report will appear shortly in the *Amer. Jour. Bot.*

The rôle of bacteria in the fouling of submerged surfaces. Claude E. ZoBell.

Studies on the sequence of events in the fouling of submerged surfaces reveal that bacteria are the predominating primary film formers. Within an hour or two after the immersion of clean glass slides in the sea, bacteria begin to attach thereto. Some of the bacteria are so tenaciously attached that they resist dislodgement when the slides are washed under running water during the staining procedures, while others are only loosely associated with the primary film. The number of bacteria increases geometrically with time until their abundance together with the simultaneous and subsequent attachment of other organisms and detritus defeat census attempts. The adsorption and accumulation of organic matter on the submerged surfaces which has been demonstrated by chemical as well as by biological procedures is believed to account for the attachment and development of bacteria, although there are other ways in which solid surfaces promote the growth of bacteria in dilute nutrient solutions.

Barnacles, hydroids, bryozoa, tunicates, algae and other fouling organisms attach to submerged surfaces coated with films of bacteria more readily than to bacteria-free surfaces. Bacteria might promote the fouling of submerged surfaces (*a*) by affording the larval forms of larger fouling organisms a foothold or otherwise mechanically facilitating their attachment, (*b*) by serving as food, (*c*) by discoloring bright or glazed surfaces, (*d*) by increasing the alkalinity of the film-surface interface thereby favoring the deposition of calcareous cements, (*e*) by influencing the e.m.f. potential of the surface or (*f*) by increasing the concentration of plant nutrients at the expense of the organic matter which the bacteria decompose.

Cell division and differentiation in living plant meristems. E. W. Sinnott.

A paper containing the material given in this talk is to be published by E. W. Sinnott and Robert Bloch under the title: "Changes in intercellular relationships during the growth and differentiation of the living plant tissues," in the *Am. Jour. Botany* for October, 1939.

AUGUST 1

The ionic permeability of frog skin as determined with the aid of radioactive indicators. Leonard I. Katzin.

The rate of penetration of ions through the excised skin of the frog was measured using radioactive isotopes as markers for the salts which had passed through. Radioactive sodium (as the chloride) was used alone and in mixtures with inactive potassium chloride; radioactive rubidium, also in the form of the chloride, was used alone and in mixture with inactive sodium chloride. Diffusion was measured for two-hour periods between isotonic salt solutions bathing the inner side of the skin, and distilled water on the outside. The results are expressed in terms of the permeability to the pure salt in each case, and parentheses used to indicate extrapolated or estimated values.

Fraction NaCl	1.0	0.75	0.67	0.50	0.25	0.10	0.0
Permeability	1.0	1.0	1.1	1.15	1.6	1.8	(2.0)
Fraction RbCl	0.0	0.25	0.33	0.50	0.75	—	1.0
Permeability	(0.5)	(0.5)	(0.65)	0.66	0.7	—	1.0

The absolute rates of passage for the pure salts are 4.7×10^{-12} mols cm.$^{-2}$ sec.$^{-1}$ for sodium chloride and 125×10^{-12} mols cm.$^{-2}$ sec.$^{-1}$ for rubidium chloride. Values for the permeability ratio of RbCl and NaCl vary between 11 and 15, but are essentially constant. These results indicate that KCl (and probably RbCl) exert small influence

on the permeability level of the frog skin, and that the passage of individual salts is essentially independent. Further, the large differences in rate of passage of rubidium and sodium ions indicates what has long been postulated, that the mobility of ions through tissues is not the same as in pure aqueous solution. An expression has been derived for the electrical resistance of the skin, from the Nernst diffusion coefficient and the Fick diffusion equation, on the assumption that the salts studied are the principal carriers of the electrical current. Sample calculations show again that there are differences in the ion mobility through the tissue.

Crystallization of myogen from skeletal muscle. Kenneth Bailey.

The main globulin component of skeletal muscle, myosin, is now well characterized. The separation of the remaining components, Weber's globulin X and the albumin myogen, has depended upon fractionation by dialysis, a procedure unsuited to many tissue proteins which denature in an ion-free solution. An attempt has been made to crystallize myogen by two methods: (1) The perfused, freshly excised minced muscle from rabbit is extracted with 10 per cent KCl at pH 7, and the solution after filtration through pulp is treated with ammonium sulphate which is dialyzed in through a collodian bag at room temperature. The precipitated myosin is filtered off when the specific gravity of the solution reaches 1.14. The solution is now brought to a pH of 6 by addition of sulphuric acid, and more ammonium sulphate is fed in until a specific gravity of 1.18 is attained; the amorphous globulin precipitates are separated, and the sulphate concentration again increased very slowly. Between a specific gravity of 1.18 and 1.22 the myogen separates in crystalline form together with some amorphous material which dissolves on cautious dilution, leaving the crystals in suspension. (2) The minced muscle is mixed with an equal volume of ice cold water and after standing for 20 minutes the juice is expressed. This is treated as outlined above.

The crystallization of myogen has recently been reported [1] by Baranowski; two crystal types were obtained, the one, termed myogen *A*, crystallizing as hexagonal bipyramids and the other, myogen *B*, as long thin plates. The *A* form is obtained by fractionation of muscle press juice by a procedure which involves a heat treatment at 50°, and the *B* form, which appears to be identical with the author's preparation, crystallizes fortuitously from the mother liquor. The physico-chemical properties of myogen *B* (purity by classical solubility methods, molecular weight, dielectric increment, titration curve) are now being investigated.

Chemical and histochemical observations on Macracanthorhynchus hirudinaceus. Theodor von Brand.

The females of *Macracanthorhynchus hirudinaceus* contain 1.13 per cent glycogen and 0.95 per cent ether extract. Phospholipids, unsaponifiable matter and unsaturated fatty acids are the chief components of the ether extract. Relatively more glycogen than fat is stored in the body-wall, whereas the contrary is true for the reproductive cells. Differential staining showed that the chief places of glycogen deposition are the hypodermis, the muscles and the mature eggs, those for the fat are the hypodermis and the ovaries.

pH reactions during feeding in the ciliate Bresslaua (Accompanied by photomicrographs taken on Kodachrome). C. Lloyd Claff and G. W. Kidder.

The large ciliate *Bresslaua*, a carnivorous member of the family Colpodidae, was used for experiments and observations on the pH condition during various phases of feeding and digestion. The general method was as follows: *Bresslaua* were treated

[1] Baranowski, *Zeitschr. f. Physiol.*, **260**, 43 (1939).

with dilute indicator dyes and were later fed untreated *Colpoda steini*, the subsequent color changes studied under the water immersion lens. When neutral red- or methyl red-treated animals were used the color changes were very striking while other indicators gave less satisfactory results.

In neutral red preparations the medium, being slightly alkaline, shows a pale yellow coloration which extends into the oral cavity. As the first *Colpoda* is taken into the mouth a secondary vacuole forms in the posterior protoplasm to receive the prey. The contents of this vacuole appears faintly pink but as the prey is trapped by a thin sheet of protoplasm cutting the secondary vacuole off from the mouth region the periphery of the vacuole is outlined by a collection of brilliant red granules or droplets from the cytoplasm of the carnivore. The prey is immediately killed. Within a few seconds the granules disappear from view. The prey does not take on any of the red coloration at this time but slowly becomes yellow, then, as digestion proceeds, through orange to a cherry red.

The conclusions reached were that the actual killing of the prey is brought about by some strong acid. A pressure system was considered but ruled out by direct observation, in that the cilia of the prey stand out very straight from the body at the moment of killing. Methylene blue-treated *Colpoda* showed no color reduction, therefore lack of oxygen as a means of killing seems improbable. The color changes in the food masses subsequent to killing indicate that digestion is alkaline in nature and that the final residuum shifts back to an acid state.

AUGUST 8

The dielectric properties of insulin solutions. J. D. Ferry.

The material contained in this talk will appear shortly in *Science* under the title: "Studies in the physical chemistry of Insulin. I. The solubility and dielectric properties of insulin and its crystallization with radioactive zinc," by Edwin J. Cohn, John D. Ferry, J. J. Livingood, and Muriel H. Blanchard.

The effects of lack of oxygen, and of low oxygen tensions, on the activities of some Protozoa. J. A. Kitching.

A comparative investigation was undertaken of the effects of lack of oxygen, of low oxygen tensions, and of some respiratory narcotics, on the activity of Protozoa. The organisms were suspended in a thin hanging drop, and oxygen-free hydrogen or nitrogen (purified over hot platinized asbestos or hot copper), or hydrogen mixed with oxygen in known proportions by means of flow meters, were flushed continually *via* lead tubing with seals of de Khotinsky cement through the observation chamber. The purity of the oxygen-free gas was checked by the extinction of luminescence of marine luminous bacteria and by mass spectrographic analysis—methods sensitive to about 0.005 mm. of oxygen and to one part in 10^5 respectively.

In the peritrich ciliate *Cothurnia kellicottiana* pure hydrogen caused an almost immediate stoppage of the contractile vacuole and cilia, and a swelling of the body; very often the body blistered. Return to air led to an immediate recovery of the vacuole to a rate of output at first much above the normal. The cilia resumed their beat, and the body slowly shrank to its original size or less. A partial pressure of 1.1 mm. of oxygen was not enough to allow any vacuolar activity; at 1.6 mm. the contractile vacuole stopped but recovered slightly after the body had swollen; and in 3 mm. there was full activity. In dilute cyanide there was little or no activity of the contractile vacuole.

In fresh-water amoebae of the "proteus" type the contractile vacuole quickly ceased all activity in absence of oxygen, or in cyanide; although amoeboid movement continued for some hours. Recovery in air from lack of oxygen was rapid. Amoeboid movement and the activity of the digestive vacuoles of the marine amoeba *Flabellula*

mira were rapidly and reversibly inhibited in absence of oxygen. Some measure of recovery from lack of oxygen was obtained at 0.3 mm.

Paramecium spp. continued swimming for some time in the absence of oxygen, but eventually stopped and cytolysed. The best survival, namely 12 hours, was obtained in culture medium and with sufficient carbon dioxide added to the hydrogen or nitrogen to maintain the pH at a reasonable value. Anaerobic survival was much shorter in dilute phosphate buffer, or for starved animals. Some recovery from lack of oxygen was obtained in 0.3 mm. oxygen.

No correlation could be found between the degree of sensitivity of Protozoa to cyanide and the critical oxygen tensions at which these organisms were just able to maintain some activity.

Nerve asphyxiation and aerobic recovery in relation to temperature.[1] Herbert Shapiro.

The sciatic nerve of the Hungarian bullfrog, *R. esculenta*, was mounted on 3 platinum electrodes for stimulating, and 2 calomel recording electrodes in an all glass chamber, which could be immersed in a Dewar flask and thus kept at constant temperature over long periods. Moistened purified nitrogen or hydrogen was passed through the chamber, and test stimuli from a commutator permitting condenser charges and discharges through the nerve at any desired frequency, were applied at regular intervals. The duration of the tetanus was controlled with a Lucas contact breaker. The total action current was integrated ballistically by a Zernicke Zd galvanometer of 4-second period. During anaerobiosis, the response of the nerve to a standard stimulus gradually decreases, and finally is extinguished. At higher temperatures "overshoot" occurs during early anoxia. After positivity disappears earlier than action current, but injury potential was never completely abolished during the experiment. Continuous tetanization shortens asphyxiation time. Time for asphyxiation of action current is an exponential function of temperature, requiring approximately an hour at 38° C., and about 1150 minutes at 0° C. The form of the function may be described by the Arrhenius equation, yielding a temperature characteristic of 11,100 calories. Evidently the nerve utilizes energy from an anaerobic reaction for setting up and conducting action currents, and this reaction runs to completion at a rate determined by the temperature. Upon readmission of oxygen to the nerve, action current, injury potential and after positivity showed recovery. Rate of recovery of action current also conformed to the Arrhenius equation with a μ value of 28,000 calories. Examination of Amberson's data for temperature effect on the absolute refractory period of nerve shows a μ value of 18,400. Underlying chemical reactions for conduction, refractory period, and recovery in nerve proposed by Gerard are such as to suggest three different types of reaction, and hence different temperature characteristics. Though the latter by themselves do not identify the reactants involved, it is of interest that the present study indicates chemical bases for these several aspects of nerve activity, with different enzyme systems acting as the controlling links.

Effects of hydrostatic pressure upon certain cellular processes. D. A. Marsland.

The experiments demonstrate that pressure induces a solation of protoplasmic gels in a number of different cells and in an inanimate gel prepared of the myosin of rabbit muscle. In each of the cells studied, the solation is associated with a retardation of movement—amoeboid movement, the pinching of the cleavage furrow, and the streaming of plant cells.

[1] This work was done during the tenure of a fellowship of the John Simon Guggenheim Memorial Foundation.

In order to see cells while under compression, it was necessary to construct a special chamber. This chamber permits objects within it to be viewed at a magnification of 600 diameters at pressures up to 600 atmospheres.

The pressure effects upon the rigidity of the cellular gels were measured by the centrifugal method. A centrifuge head was constructed which permits centrifugation during the period in which the cells are under compression.

When amoebae are suddenly exposed to a relatively high pressure (above 450 atmospheres) a peculiar reaction suddenly occurs. Each of the extended pseudopodia collapses and tends to round up. This result would be expected if the plasmagel, which supports the pseudopodia, were to undergo liquefaction. If the pressure is maintained for a few minutes, the whole cell becomes spherical. However, when the pressure is released, active amoeboid movement begins again within a minute or so.

Centrifuge experiments demonstrate that the form of the amoeba is related to the structural properties of the plasmagel. When the amoebae are centrifuged under pressure, the liquefaction is indicated by the rapidity with which the granular components of the plasmagel undergo displacement. In the higher range of pressure, the rigidity of the plasma gel is reduced to a small fraction of the normal value. In this range of pressure, no pseudopodia may be sustained. In the lower range, pseudopodia may be formed, but they display a graded diminution of diameter and length as the pressure is increased.

Comparable results were obtained in studies of cleaving *Arbacia* eggs and the streaming of the leaf cells of *Elodea*. The retardation of the furrowing parallels the loss of rigidity of the gelated cortex of the egg, and the streaming of the *Elodea* cell is slowed in proportion to the degree of liquefaction which occurs in the non-flowing part of the protoplasm. Thus it appears that sol-gel reactions are providing a machine by which the cell can transform chemical potential energy into mechanical work.

AUGUST 15

On the nature of the material elaborated by fertilizable Nereis eggs inducing spawning of the male. Grace Townsend.

The observations of Lillie on the spawning reaction of *Nereis* limbata led to the formation of the "fertilizin theory" (Just, 1930). Lillie found evidence that the material inducing spawning of the male came only from fertilizable eggs.

I have re-investigated the relation of the spawning inducing material to fertilization and have found it to possess properties in common with material essential to egg activation though not necessarily associated with the phenomenon of egg and sperm union.

Egg-cell activation may plausibly involve processes common to all species and be based on the same processes as may initiate cell division in any tissue. Correspondingly, spawning was induced by extracts of many fresh tissues from many species, all of which contain glutathione. Crystalline pure glutathione in one part in a million in a single drop quantity, and the molecular constituent, cystine or cysteine, in higher concentration, induced the natural spawning reaction.

Marine eggs are rich in glutathione (300–700 mg. per 100 gm. wet weight) and it is especially concentrated in the germinal vesicle. A reducing substance passes from *Nereis* eggs. The reaction of the male to glutathione, by a large variety of tests, was indicated to be extremely specific.

All substances found to destroy the spawning inducing property of egg-water and glutathione inhibit fertilization.

Properties of Egg-Water Spawning Inducing Material and Glutathione and "Fertilizin" and Its Described Fractions

Analysis of properties:	Egg-Water Spawning-Inducing Material	Glutathione	Woodward's		Lillie's Fertilizin
			Parthenogenetic Fraction	Agglutinin Fraction	
Elaboration from egg	+	+	+		+
Xanthoproteic test	−	−	−		−
Stability in acid	+	+			
Stability in alkali	−	−			
Precipitation by acetone	+	+	+		
Berkfeld filter	+	+	+	−	−
Dialysis	+	+	+	−	−
Inactivation by aging	+++	+	+++	+	+
Inactivation by boiling in s. H_2O	+++	+	+++	+	+
Inactivation by irradiation	+++	+	+++	+	+
Inactivation by Au, Cu, Ni, etc.	+++	+	+	−	+
Inactivation by CH_2ICOOH	+++	+			
Inactivation by KCN	−	−			−
Inactivation by blood, coelomic fluid	+	+	+		+
Inactivation by cytolyzed eggs	+	+			+
Inactivation by cytolyzed tissues	−	−			
Induction of spawning	+	+			+

Ovum and spermatozoön age at the time of fertilization and the course of gestation and development in the guinea pig. W. C. Young.

An abstract of this paper has already been published in the *Anat. Rec.* by Arnold F. Soderwall, William C. Young and Richard J. Blandau, under the title: "Spermatozoa vitality in the genital tract of the guinea pig" (*Anat. Rec.*, vol. 73, Suppl. 2, p. 47, 1939).

Experiments on the production of haploid salamanders. Cornelius T. Kaylor.

The eggs of many species of amphibians can be induced to begin their development with only one set of chromosomes. This would then produce haploid embryos and larvae.

In spite of the large number of experiments which have been performed on the production of haploid amphibians (see review of Fankhauser, *Jour. Hered.*, 28, 1937), the results have varied with the species used, the degree of abnormality of embryos produced, and with the extent of development. So far as demonstrated, only one completely haploid amphibian has been reared to a stage approaching sexual maturity (Baltzer and Fankhauser, 1922). This is in striking contrast to the fact that normal haploid animals exist in nature, as well as to the fact that viable haploid plants have been produced experimentally.

The present experiments were undertaken primarily to test, by new methods, with the eggs of species which have not been used extensively before, the possibilities of advanced haploid development in these species. Two species of newts were used: *Triturus viridescens* and *Triturus pyrrhogaster*. The female chromosomes were removed from the egg with a small pipette. All subsequent development then took place by means of the male chromosomes.

In over 200 experiments with the eggs of *T. viridescens*, only about 15 per cent of the embryos developed beyond the gastrula stage. In 76 experiments with pyrrhogaster eggs, about 30 per cent of all embryos developed to stages ranging from a neurula to a 120-day-old larva. This larva, however, was not completely haploid. Another larva, which in external appearance resembles all haploid larvae which have been produced in other experiments, was fixed at 47 days of age, at a time when hind limb buds had appeared. It had a deformed jaw and could not take food.

It appears, then, that for the purpose of obtaining advanced haploid larvae in these amphibians, the eggs of *Triturus pyrrhogaster* are much more adaptable than eggs of *Triturus viridescens*.

Regulation in mosaic eggs. Alex B. Novikoff.

Transplantation experiments on the eggs of *Sabellaria vulgaris* by the author [1] have demonstrated that the materials in the polar lobe which are involved in the formation of the apical tuft and the post-trochal region do not diffuse from the transplanted lobe into adjacent cells. But there were some indications that when the polar lobe material was incorporated into a cell the course of development of the cell was altered.

A 7.5 per cent solution of 2.5 Normal KCL in sea water inhibits cleavage in the eggs of *Sabellaria*. Eggs are placed into the solution after the second maturation division, and kept there until the controls have completed the first cleavage. Upon returning them to sea water, as many as 90 per cent of the eggs develop into perfect double embryos. These embryos possess two eye spots, two sets of posttrochal bristles, two posterior cilia, two sets of dorsal cilia, two neurotrochs, two intestines, one central stomach, probably one oesophagus, one mouth, and two mouth folds.

At least some of the cells in these larvae have developed into structures which they do not form in normal development. Thus the prospective potency of these cells is revealed to be wider than the prospective fate. This is a characteristic generally associated with regulative eggs.

Eggs placed in the KCL solution *after* the completion of the first cleavage until the controls have completed the second cleavage do not develop into double embryos. Instead larvae which possess extra bristles, or extra eye spots, or both, are produced.

The formation of double embryos suggests that the material of the first polar lobe has been distributed to each of the first two blastomeres where it 'organizes' in each a new embryonic axis.

August 21

Micromanipulative studies. (*Motion picture*). Robert Chambers.
Living cells in action (*motion pictures*). C. C. Speidel.

Ciné-photomicrographs of the fast motion type have been taken of many kinds of cells. The pictures are made directly from living frog tadpoles and they reveal characteristic cellular movements and reactions under normal and experimental conditions.

The pictures include examples of the growth, migration, mitosis, and differentiation of connective tissue cells, epithelial cells, vacuolated sub-epidermal cells, endothelial cells of blood and lymph capillaries, sheath cells, regenerating spinal cord cells, pigment cells, and various kinds of leucocytes. A complete record of nerve regeneration over a period of a month is given, including the stages featured by growth cones, sheath cells, and myelin segments.

Case histories are also presented to show the changes in position from day to day of the relatively stable cutaneous nerve endings which belong to myelinated fibers. These include examples of extension, retraction, irritation, autotomy, and new growth cone differentiation following loss by phagocytosis. Several cases are given which

[1] *Biol. Bull.*, 1938, **74**, p. 211.

reveal how red blood cells that have been extruded from blood vessels are engulfed by macrophages.

Various types of behavior of localized contraction nodes in single muscle fibers (from *Palaemonetes* leg and *Limulus* heart) are also presented. These include their formation, progression, splitting, reflection, collision, and dissipation; also their progression past thin clots resembling intercalated discs.

Other pictures (obtained with the coöperation of Dr. Ethel Harvey) show the early developmental history of the sea urchin, *Arbacia*, including the immature egg, mature egg just before and just after fertilization, segmentation stages from 1 to 64 cells, free-swimming gastrula, and pluteus. Other pictures show abnormal cleavages of centrifuged eggs and of the clear halves of centrifuged eggs.

Polariscopic pictures reveal the birefringent substances in pigment cells, epithelial cells, muscle fibers during contraction and relaxation, and in the developing eggs and larvae of *Arbacia*.

AUGUST 22

Studies on the life history of Spelotrema Nicolli. R. M. Cable and A. V. Hunninen.

Metacercariae occurring in the blue crab, *Callinectes sapidus*, were fed to six young herring gulls which were killed and examined 12 hours to 18 days later. Each bird yielded a large number of adult *Spelotrema nicolli* while six controls were negative for this species. Since very young metacercariae indicated clearly that the cercaria is of the Ubiquita type, an extensive search was made for such a larva in mollusks occurring where infected crabs were abundant. After many unsuccessful attempts, a cercaria of the type sought was found in the snail, *Bittium alternatum*. This cercaria was found to enter the crab by way of the gills, passing with the circulation to strands of muscle-like tissue where encystment occurred. The morphology of various stages in the life history of *S. nicolli* has been studied in detail; the excretory formula of the cercaria is $2[(1 + 1) + (1 + 1)]$, the metacercaria and adult, $2[(2 + 2) + (2 + 2)]$.

Stabilizing action of alkaline earths upon crab nerve membranes, as manifested in resting potential measurements. Rita Guttman.

Resting potentials of the non-myelinated nerve of the proximal segment of the claw or of the first walking leg of the spider crab, *Libinia canaliculata*, were measured by means of a potentiometer and null point galvanometer. All solutions used were approximately isotonic with sea water and pH was controlled.

It was found that the alkaline earths, in themselves, have no effect upon the magnitude of the potential. Yet they are able to prevent the usual depressing action of KCl upon the potential when the alkaline earth and KCl are simultaneously applied to the nerve. Solutions containing two parts of $BaCl_2$ to one part of KCl, five parts of $SrCl_2$ to one part of KCl, and eleven parts of $CaCl_2$ to one part of KCl are threshold values for the neutralization effect. The order of effectiveness of the alkaline earths for counteracting the depression of the potential by KCl is thus Ba, Sr, Ca. This is the order in which these elements appear in the atomic table.

The alkaline earth, Ba, is also capable of preventing the depression of the resting potential by various lipoid-soluble, surface active, highly polar substances, viz., veratrine sulphate, chloral hydrate, iso amyl urethane, sodium salicylate and saponin.

The neutralization effect of the alkaline earths may last over a period of many hours. The alkaline earths are capable of preventing the action of depressants strong enough to cause, when present alone, a decrease of potential of fifty per cent or more.

A direct relationship was found between the amount of depression of the potential per unit time caused by veratrine sulphate and the logarithm of the concentration of veratrine sulphate acting. Although a concentration effect is obtained with both K and veratrine sulphate, it is not necessary to assume that the action of these substances upon the nerve is identical. It should be noted that the relative concentration of KCl (one part isotonic KCl to eleven parts sea water, or 0.04 M) necessary to depress the potential is of a much greater order of magnitude than that of the organic substances (0.00004 M veratrine sulphate in sea water).

Two possible explanations for the neutralizing action of the alkaline earths are: (1) change of effective pore size in a sieve-like membrane by the alkaline earths or (2) alteration of the partition coefficients of the depressants by the alkaline earths. There is at present no basis for deciding between these alternative concepts. The phenomenon is certainly, however, not one of antagonism in the classical sense of Jacques Loeb, inasmuch as, for one thing, the quantities of alkaline earth necessary are much too large.

An experimental study of the pigment granules of the Arbacia egg. D. L. Harris.

The pigment granules are isolated by breaking eggs in 0.35 m Na_3 citrate to avoid the presence of Ca. Unbroken cells are removed by centrifuging. It is found that the granules are actually vacuoles. They are easily deformed, and regain spherical shape when external force is removed. In the presence of Ca, Mg, and Sr; hypotonic solutions; and isotonic solutions of urea, acetamide, and ethylene glycol they discharge pigment. Moreover, in Ca, Mg, and Sr solutions, contiguous granules coalesce to form fluid vacuoles containing small granules in active Brownian movement.

The discharge of pigment in hypotonic solutions was investigated quantitatively. The number of granules present at various concentrations of Na_3 citrate was determined by counting. It was found that there was much more extensive breakdown at low concentrations. Furthermore, there is a normal distribution of granules in respect to ease of breakdown.

In the absence of Ca, Mg, or Sr, the pigment outside of the granule turns greenish brown and finally green. A suspension of granules in Na_3 citrate treated with hypotonic solutions will therefore change color from pink to green. The amount of granule breakdown may be determined by comparing the color in a given hypotonic suspension with standards prepared by making mixtures of pink suspension and green suspension. This method gives results in agreement with those of the counting method.

The rate of granule breakdown in various hypotonic concentrations was determined by measuring the time to reach a given color, *i.e.*, a given amount of breakdown. Using eight end-points, it was found that as the concentration was decreased the rate of reaction increased. This is clear evidence that the granules behave as osmometers. It is concluded that the granules are vacuoles surrounded by a semi-permeable membrane, and that they do not constitute osmotic dead space within the cell.

The action of calcium on muscle protoplasm. L. V. Heilbrunn.

The complete paper is in press and will appear shortly in *Physiol. Zool.*

General Scientific Meetings

August 29

The mechanism of membrane elevation in the egg of Nereis. D. P. Costello and R. A. Young.

Certain agents may induce the formation of a very wide perivitelline space in the *Nereis* egg. Alkaline NaCl (pH 10.5) was used by D. P. Costello, and X-rays (8,800–41,300 r) were used independently by R. A. Young.

When placed in alkaline NaCl the uninseminated egg elevates the vitelline membrane (occasionally double), and the width of the perivitelline space increases until the membrane ruptures, setting free the ovum. It was demonstrated by means of a Chinese ink suspension and by microneedles, that there is no jelly external to the intact membrane, but that jelly fills the perivitelline space and escapes with the ovum upon membrane rupture. Attempts to inseminate such eggs after return to sea water have been unsuccessful. Alkaline NaCl is a parthenogenetic activating agent. If normally inseminated eggs are subsequently treated with alkaline NaCl, the passage of cortical jelly through the membrane ceases, and a perivitelline space appears of width inversely proportional to the jelly already extruded. Protoplasmic cone-shaped filaments, temporarily adhering to the elevating membrane, are gradually retracted, except the one to which the activating spermatozoön is attached. The spermatozoön passes through the membrane 7–10 minutes after the egg is introduced into alkaline NaCl. The penetration of the sperm into the treated egg takes place three times as rapidly as under normal conditions, and can be followed with unprecedented clarity.

If *Nereis* eggs are treated with X-rays, and inseminated after 5–10 minutes, a similar, but much slower, response is induced. The width of the perivitelline space is directly proportional to the dosage, whereas the extrusion of jelly through the membrane is inversely proportional to dosage. In these eggs the sperm frequently does not complete the penetration process.

The colloidal nature of the perivitelline material is demonstrated by collapsing the elevated membrane against the egg surface with a solution of gum arabic in sea water.

It is suggested that exaggerated membrane elevation of the *Nereis* egg may be obtained by agents which (1) initiate the outflow of the cortical jelly precursor (if the egg has not been previously inseminated), and which (2) alter either the vitelline membrane or the jelly in such a way that passage of the jelly through the membrane is completely or partially prevented.

Determination and induction of the anuran olfactory organ. Edgar Zwilling.

By means of large transplants of presumptive head ectoderm from gastrulae of varied ages to the flank of older urodele embryos it was established that the olfactory organ of *Rana pipiens* is determined (*i.e.* would self-differentiate) before the neural folds are present. This determination probably occurs in the small yolk-plug stage. The presumptive olfactory material of pre-neurula stages and of early neural plate stages self-differentiated in the absence of brain tissue. Since it was possible that the presumptive brain tissue might act laterally upon cells in the same layer and so be responsible for the determination, the brain tissue was tested for its inductive capacity. Forebrain material from various stages during neurulation was transplanted beneath the epidermis of the flank region of various host stages. In only one case did olfactory material develop; and since this operation was an heteroplastic one (to *Rana palustris*) it could be determined that the olfactory tissue was of donor origin. The anterior portion of the roof of the archenteron was then implanted into

the blastocoeles of older gastrulae to test its inducing power for this structure. Of 19 surviving cases 5 developed small but perfect heads—which had olfactory organs. One of the others developed an olfactory organ in the absence of brain. Of 9 controls (where forebrain of the open neural plate stage was implanted into similar gastrulae) one developed an olfactory canal. The origin of this tissue cannot be established with certainty since this was an homoplastic graft.

A method of determining the sex of Arbacia, and a new method of producing twins, triplets and quadruplets. Ethel Browne Harvey.

To determine the sex of *Arbacia*, inject a few drops of a saturated solution of NaCl in sea water into the mouth; then with a fine pipette inject a little of the same solution into *one* genital pore. A few eggs or a little sperm will exude *only* from the opening injected, easily distinguishable by color. If thrown immediately into a jar of still sea water, the animal will stop shedding, and the gonads remain normal and intact. If the animal does not respond to NaCl, a molar solution of KCl can be used; this is more drastic.

To obtain twins, place the eggs from which fertilization membranes have been removed in a solution of 30 grams of NaCl per liter of sea water for 5 to 10 minutes, immediately after first cleavage; then remove to sea water. The first two blastomeres are nicely separated, but connected by a thin strand, and they develop independently. The blastulae at first swim in pairs and can easily be isolated. Some pairs gave both normal plutei, some both abnormal, and some, one normal one abnormal. In lots of 10 pairs, development was variable, but in one lot all but one became perfect or almost perfect plutei. Quadruplets are obtained by similar treatment just after second cleavage; four almost perfect plutei have developed from the first four blastomeres in some cases. Triplets are obtained when two of the blastomeres of the 4-cell stage develop independently, and the other two together.

An artificial nucleus in a non-nucleate half-egg. Ethel Browne Harvey.

The non-nucleate half-eggs of *Arbacia punctulata*, obtained by centrifugal force, have been injected (by W. R. Duryee) with yeast nucleic acid and thymus nucleic acid. The injected drop of fluid does not disperse through the cytoplasm, as does sea water, but remains distinct in a small drop or vacuole surrounded by a film or membrane, resembling strikingly the appearance of the nucleus in the living cell. The possibility of supplying the non-nucleate eggs with chemical materials found in the normal nucleus opens a new line of investigation in the effort to cause the parthenogenetic merogones (*i.e.* activated non-nucleate egg fractions) to develop beyond the blastula. Nucleic acid, nucleo-proteins, adenine, guanine, uracil, auxins, tobacco mozaic virus and many other substances, added to the medium before, during and after centrifugation have been found to have no effect.

Color responses of catfishes with single eyes. G. H. Parker.

As a rule fishes with only one eye respond by color changes to differences in their environment as successfully as do those with two eyes. In this respect the trout has long been known to be peculiar for on the loss of one eye it darkens contralaterally. The common catfish, *Ameiurus nebulosus*, when deprived of one eye conforms neither to the general rule for fishes nor to the special one for the trout. A one-eyed catfish is at first very dark, after which it may change slowly in tint according to its environment though without ever becoming fully pale. Such fishes may finally assume in the same environment somewhat different tints and retain these with considerable individual persistence. This diversity appears not to be due to variations in the irritability of the orbital wound which might influence to various degrees the stump of the optic nerve. The cause of these more or less characteristic color differences is unknown. In these respects one-eyed catfishes are unlike any other fishes thus far

described. The fact that intermedin, the secretion from the pituitary gland, plays a very important part in the color changes in catfishes, and that in this fish chromatophoral nerves, both dispersing and concentrating, are less significant than the pituitary gland, may be the occasion of the difference between *Ameiurus* and most other teleosts whose chromatophores are often under almost exclusively nervous control.

A vibration sense in a swarming annelid. Grace Townsend.

During sexual metamorphosis of *Nereis limbata* a vibration sense becomes highly developed which equips the worms for orientation in rapid swimming. The sense enables the worms, typically, to avoid striking solid objects, to relate themselves to smooth surfaces and to the opposite sex. Circling can be induced by the vibration sense alone and the sense doubtless supplements the chemical sense (Lillie and Just, 1913) in the spawning integration of this species. The marked development of the sense is associated with the metamorphosis of the dorsal and ventral cirri.

During metamorphosis the cirri become elongated beyond the surrounding parapodial structures and equipped with budding elevations, or processes, along the inner surfaces bordering the parapodial lobes. The elevations are filled with nerve cells which connect with clusters of turgid cytoplasmic hairs.

If the cirri are clipped from the parapodia of the metamorphosed worms (with use of iridectomy scissors), the worms lose their characteristic orientation to surfaces and swim unrelated to surfaces as does the non-sexual phase.

The sensory structures of the dorsal and ventral cirri are part of the lateral line system of annelids elaborately described for the Capitellidae by Eisig (1879) and for all groups by Jeener (1928). Treadwell (1900) describes a striking sensory organ on the dorsal cirrus of a swarming palolo (*Eunice auriculata*). No work has been done as to the general functional significance of the system. Stolte (1932) observed that the dorsal cirri of *Glycera* are not stimulated by chemicals or tactile stimuli but are stimulated by slight mechanical oscillations of the structures.

The plausibility of a generally distributed vibration sense in annelids possessing a pelagic phase is suggested.

Food habits of Endomoeba muris. D. H. Wenrich.

These studies were made on prepared smears of the caecal contents of rats and mice, mostly fixed with Schaudinn's fluid and stained with iron alum haematoxylin.

Endamoeba muris feeds on a great variety of food materials including various kinds of bacteria, *Blastocystis*, yeasts, filamentous organisms, starch grains, *Trichomonas*, *Giardia*, *Hexamitus*, leukocytes and erythrocytes. On the same slide some individuals may be engorged with one kind of food while others will be engorged with different kinds; still others may contain a variety of food materials. There is a tendency for the amoebae from one particular host to show food preferences different from those from some other host. For example, on one set of slides about 60 per cent of the amoebae contained a certain kind of colonial bacteria, while on another set of slides about 75 per cent showed one or more individuals of *Trichomonas muris* in their food vacuoles.

In ingesting different kinds of food objects, *E. muris* employs somewhat different methods. Fairly large starch grains are apparently surrounded by slowly advancing pseudopodia on all sides in close contact with the grain, but one pseudopodium may be further advanced than the others. The edges of the advancing pseudopodia often stain intensely with iron alum haematoxylin. Some food cups were surrounded by a dense layer of cytoplasm which sometimes did and sometimes did not stain intensely. Ingestion cones were extended along plant filaments, which became coiled inside the cell. In most cases an ingestion cone included a definite pharynx-like structure with deeply-stained walls and annular thickenings. Similar pharynx-like tubes formed without protruding cones were employed in ingesting *Trichomonas*

muris. Comparable tubes were also found deep in the cytoplasm apparently being used to break up food masses into smaller units.

A quantitative study of the hemopoietic organs of young albino rats. J. E. Kindred.

A study has been made of the differential cell counts and mitosis rates in the parotid lymph nodes, spleen, thymus and bone marrow of 15-day-old and 20-day-old albino rats. These data have been used in conjunction with differential cell counts of the blood and with the volumes of the several organs in an attempt to find out what degree of relationship exists between cell production and differentiation in the organs and the blood. Other investigators have suggested that in order to meet the demands of granulocytopoiesis and erythrocytopoiesis, the bone marrow must filter out small lymphocytes from the blood stream. The present data show that the lymph nodes and spleen are deficient in the production of small lymphocytes needed for the growth of these organs between the 15- and 20-day stages. On the other hand, the bone marrow and thymus produce an excess of small lymphocytes during this period. The excess small lymphocytes more than balance the needs of the lymph nodes, spleen and blood. The principal reason for the excess production of small lymphocytes (hemocytoblasts) in the bone marrow is an actual decrease in numbers with time, whereas in the thymus, the excess is caused by a very high mitosis rate in the peripheral zone of the cortex.

On the histology of the mammalian carotid sinus. William H. F. Addison.

The carotid sinus is the dilated beginning of the internal carotid artery at the bifurcation of the common carotid artery into the internal and external carotid arteries. The wall of the carotid sinus has the three layers characteristic of arteries, but its structure differs from that of adjoining regions of the arteries with which it is continuous by the presence in the tunica media of a predominant amount of elastic tissue and by the absence or the small amount of smooth muscle. In the sinus of the dog the elastic tissue is arranged in 8–10 coarse lamellae with collagenous tissue interspersed. The transition in structure from the sinus to the internal carotid artery is abrupt and there is a conspicuous difference in the organization of the walls. The diameter of the sinus is over twice as great as that of the adjoining internal carotid artery in the living animal. Ordinarily the carotid sinus may pass unnoticed in the dead animal because the lowering of blood pressure at death allows the sinus to diminish in size until it is only slightly larger than the diameter of the internal carotid artery. A type of carotid sinus similar to that in the dog has been seen in other mammals, *e.g.*, newborn child, rhesus monkey and cat.

On Clark's theory of muscular contraction. Alexander Sandow.

The theory of contraction for striated muscle proposed by Clark (*Am. J. Physiol.*, V. 82, p. 181, 1927) depends on the following assumptions: (1) the muscle fibers consist of alternately arranged isotropic and anisotropic layers which run unbroken across each fiber; (2) upon stimulation chemical changes occur which transform the liquid crystals of the anisotropic discs into a more solid crystalline state, while the substance of the isotropic layers remains unaltered; (3) the new relation between the substances of the layers results in the sudden production of a tension that can be calculated by means of the formula $F = 2AT/d$ where F = the force per fiber, A = the area of the fiber cross section, T = the surface tension of the material of the isotropic layers, and d = the thickness of this layer. Using the values for the frog sartorius: diameter of fiber = 50 μ, T = 70 dynes/cm., d = 0.7 μ, Clark finds F = 39.25 dynes, in fair agreement with the experimental value for maximal isometric

tension of 55 dynes (Hill, 1926). Consideration of more recent work (Hill, 1938) and allowance for Fenn's chloride space lead to the value for F of 37.5 dynes, in striking agreement with Clark's calculated value. Although muscle physiologists have paid little attention to this theory, it has received some notice in physiological literature. (Evans, 1931; von Muralt and Edsall, 1930; Burns, 1929; Howell, 1936; Barnes, 1937.)

Clark's theory may be questioned on general grounds: the assumed value of T is probably too high; there is a possibility of contradiction between the predicted and observed changes in birefringence during contraction; the assumed mechanism for tension production is itself open to criticism; and even if it be accepted it cannot be applied to unstriated muscle. The theory is definitely at variance with observation in indicating that during contraction the isotropic bands shorten relative to the anisotropic bands. But difficulties of a decisive nature arise if, conforming to the generally accepted view (Schmidt, 1926; Hürthle, 1931; Chambers and Hale, 1932; von Muralt, 1933) account is taken of the fibrillar structure of the muscle fiber. Clark's method for calculating the tension must then be applied to the individual fibrils, and the sum of their tensions taken as the tension developed by the whole fiber. Now, however, the previously used formula is not valid, since the diameter of the fibril is of the same order as the thickness of the isotropic segment of a sarcomere. The correct formula for the tension per fiber is $F = 2nAT(1/d - 1/D)$, where n = the number of fibrils per fiber, A = the cross-sectional area of a fibril, d = the thickness of the isotropic segment, D = the diameter of the fibril, and T is as before. Using Clark's values for T, d, and the diameter of the fiber, and taking $D = 1\ \mu$ and n the value for closest packing of the cylindrical fibrils, it is found that $F = 10.6$ dynes. This is clearly far too low in comparison with the experimental value to support the theory. Moreover, the assumption that $d = 0.7\ \mu$ is open to question. The work of Buchthal, Knappeis and Lindhard (1936) has shown that d in the frog sartorius may vary from 0.81 μ to 1.10 μ depending on the degree of stretch of the muscle. If $d = 0.9\ \mu$, then $F = 2.8$ dynes. And if $d > 1\ \mu$, or if in general $d > D$, then F becomes negative indicating instability of the system and the separation of the fibrils into discrete sarcomeres. In view of the difficulties and inadmissable implications of Clark's theory we must conclude that it cannot be accepted as a valid picture of the mechanism of contraction of striated muscle.

Conditions governing the frequency of contraction of the heart of Venus mercenaria. Albert E. Navez and John D. Crawford.

Two characteristics: frequency and amplitude of the beat of the excised and perfused ventricular portion of the heart have been studied. This note is concerned only with the frequency.

If one plots against time, the frequency of the heart perfused with non-aerated sea-water, one obtains a curve showing a rapid rise (in about 2 hours) to a maximum frequency followed by a slow decline to zero in about 36 hours. Perfusion with aerated sea-water determines a frequency curve which reaches a plateau, slightly below the maximum value and which extends over 36 or more hours before final decline sets in. If a solution of one part of dextrose into 250,000 parts of well aerated sea-water is used as perfusion liquid, the plateau of constant frequency may be extended to 72 to 96 hours. The rate of perfusion is sufficient when above 20 ml./min.

Frequency is a function of load (tension) on the heart. The load determines the time elapsing between excision and maximum in frequency curve. In general less time is required to reach maximum frequency under greater loads. The graph of "time of excision to time of maximum frequency" against "load" is a hyperbola with a short induction period for loads below 30 mg. The "excess frequency" (frequency above that of the level plateau mentioned above) is also a function of the load. The value of the "excess" frequency is greatest at about 80–95 mg. load and is of smaller value for loads above or below this point.

For a heart beating at constant frequency under a given load, any change in load below a critical tension of about 200 mg. effects a change in frequency that is completely reversible. When the load on a heart is changed, the frequency changes correspondingly, reaching a new value through a series of "damped oscillations" over a period of more than an hour at room temperature. Above the critical load, changes in frequency are not exactly reversible. On passing from 100 mg. to 250 mg., the frequency rises; on return to 100 mg. the frequency falls decidedly below its original value.

In all experiments reported the temperature was kept constant to within $\pm 0.01°$ C.

Small variations in temperature affect decidedly the rate of contraction. Four (or five) temperature (characteristics) have been found for a large series of hearts studied. Critical temperatures have been found around 10°, 15°, 20–21°, and 25°; not all appear in every heart studied.

The refractory period in the non-conducted response of striated muscle. F. J. M. Sichel.

In the isolated skeletal muscle fibre (frog), with cut ends, the contractile mechanism can be excited to a normal type of response apparently without involving the conductile mechanism (Brown & Sichel, *J. C. C. P.*, **8**, 315, 1936; Sichel & Prosser, *Biol. Bull.*, **73**, 293, 1937). This non-propagated response has no absolute refractory period and the size of the response is a function of the strength of the stimulus even though the entire length of the fibre is involved in the contraction.

During the course of an extension of these experiments in collaboration with D. E. S. Brown use was made of the fact that twitch-like contractions without propagation can also be obtained in the intact entire sartorius provided the KCl content of the medium is 70 mg. per cent. If the entire muscle is stimulated under these conditions by means of massive electrodes so placed that the electrical field is at right angles to the longitudinal axis of the muscle, the entire muscle is involved in the contraction. Recorded isometrically these contractions have a normal form. They resemble the contractions of the isolated fibre preparation and differ from those of the normal muscle in that they are essentially local and non-propagated and also in that no refractory period is involved in their excitation. The absence of the refractory period was demonstrated as in the case of the isolated fibre preparation by stimulating with two equal condenser discharges separated by a variable time interval. For the whole muscle stimuli of rectangular form were also used. In the isolated fibre and in the KCl-treated muscle the second stimulus will always contribute something to the mechanical response; in the normal muscle there is an interval, related to the absolute refractory period, during which the second stimulus can contribute nothing. Since the response of the KCl-treated muscle is non-propagated, its grading presumably does not necessarily involve the frequency distribution of the fibre thresholds.

Pigment inheritance in the Fundulus-Scomber hybrid. Alice M. Russell.

Hybridizations between *Fundulus heteroclitus* ♀ and *Scomber scombrus* ♂ were made successfully during June and July, 1938 and 1939 at Woods Hole. The abnormality of the embryos, and the appearance of paternal pigmentation in the hybrid, as previously described by H. H. Newman, were confirmed.

A systematic re-investigation of the inheritance of pigmentation, followed by a cytological and morphological study of the hybrid seemed worthwhile.

Development of pigmentation in normal *Scomber scombrus* is described for the first time. Tables comparing the number of melanophores on embryos, and yolk, in parents and hybrids, seem to reveal a *Scomber*-effect in the hybrid.

As regards the chromatophores, the hybrid embryos appear to fall into three categories: those with *Scomber* type, those with *Fundulus* type, and those with both types of chromatophores. The melanophores and chromatophores in parents and hybrids are distinguishable: those in the hybrid being identical in color and structure with those of the parents. However, a close and systematic study of the hybrids reveals an enormous variability of combinations of the parental pigment cells. Actually, no two embryos are identical in their pigmentation pattern.

This enormous variation found in the F_1 generation is unusual, and at present inexplicable. The reciprocal cross was never successfully made.

The use of the swimbladder by fish in respiratory stress. Virginia Safford.

It is generally agreed that the chief function of the swimbladder is to maintain buoyancy in the fish. The use of oxygen in the swimbladder for respiration has been shown by Potter for physostomous fish (*J. Exp. Zool.*, **49**, 45, 1927). Physoclistous fish when confined in a limited volume also show gaseous exchange in the bladder without change in external pressure on the fish.

The scup, sea robin, cunner, tautog, fundulus and toadfish (all physoclisti) were bottled by the method of Fry and Black (*Am. J. Physiol.*, **126**, p. 497, 1939) in water with various pressures of CO_2. The water was analyzed for CO_2 and O_2 at the death of the fish and the results showed a characteristic curve for each species, *i.e.* the ability to use oxygen decreases with the increase in pressure of CO_2. At the same time gas samples were taken from the swimbladders and analyzed for CO_2 and O_2 by the use of Krogh's micro-gas-analyzer.

The analyses showed that for these species: (1) CO_2 passes freely into the swimbladder, the fish equilibrating with external CO_2 up to the point where his ability to use oxygen in the water decreases. (2) Oxygen in the bladder is used at low CO_2 pressures in the water but the ability to use oxygen in the bladder decreases with the rise in CO_2 in a manner parallel with the ability to use oxygen from the water.

Species differences occur in the curves showing the ability of the fish to use oxygen from water and swimbladder in the presence of CO_2. The ratio of CO_2 in the water and swimbladder is practically constant for each species, *i.e.* $\frac{\text{swimbladder } CO_2}{\text{water } CO_2} = k$, up to the point where utilization of oxygen in the water decreases. The values of k in these six species were not very different.

It seems, therefore, that the same sort of mechanism effects gaseous exchange between swimbladder and blood and between water and blood in the fish under the conditions of the experiments described.

Water permeability of Chaetopterus eggs. Herbert Shapiro.

The permeability of the egg of the annelid worm, *Chaetopterus pergamentaceus*, to water was determined by allowing the egg to swell in 60 per cent sea water. The equilibrium volumes of about thirty eggs were measured at various dilutions of sea water, and found to conform to Boyle's law. From the equation

$$P_0(V_0 - b) = P_{ss}(V_{eq} - b)$$

(where V_0 and P_0 represent respectively the egg volume, and osmotic pressure in normal sea water, and V_{eq} the cell volume at equilibrium in diluted sea water of osmotic pressure P_{ss}), the "osmotically inert volume," b, was found to be about 34 per cent of the cell volume. Individual plots were made of the kinetics of osmosis of 27 unfertilized eggs and of 21 fertilized eggs (at room temperatures, 22 to 25° C.). The permeability constant, K, was calculated for the first, third and fifth minute of swelling from the relationship proposed by Lucké, Hartline and McCutcheon (*Physiol. Rev.*, **12**, 68, 1932) viz.,

$$dV/dt = KS(P - P_{eq}),$$

where dV/dt represents the rate of change of egg volume at any instant, S the surface area of the cell, $P - P_{eq}$ the difference in osmotic pressure between the interior and exterior of the cell, and K the cubic micra of water passing across a square micron of cell surface per minute per atmosphere osmotic pressure difference. The "osmotically inert volume" of each egg was taken into consideration. These values for the unfertilized eggs obtained by direct microscopic measurement ($K = 0.5$) agree with those previously reported by Lucké, Ricca and Hartline (*Biol. Bull.*, **71**: 397, 1936) using a diffractometer method. It was also found that the permeability of the *Chaetopterus* egg to water shows little change on fertilization, the data indicating a slight increase ($K = 0.6$). Whitaker (*Jour. Gen. Physiol.*, **16**: 475, 1933) has demonstrated a decline in respiration of the *Chaetopterus* egg when fertilized. Thus it is evident that the parallelism between alterations in respiration and water permeability, which appeared on fertilization of the eggs of *Arbacia* and *Asterias*, is not a universal feature of the activity of marine eggs.

A mechanism of increased cell permeability resembling catalysis. M. H. Jacobs and A. K. Parpart.

It was shown by Ørskov that the rate of entrance of NH_4Cl into the mammalian erythrocyte is greatly accelerated by low concentrations of bicarbonates. This effect was attributed to an increased permeability of the cell to the NH_4 ion. The following is suggested as a more plausible explanation. NH_4Cl enters the erythrocyte by the penetration of NH_3 followed by the exchange of OH for Cl ions at a rate that depends on the value of $[OH]_i[Cl]_o - [OH]_o[Cl]_i$. This rate is relatively slow because of the low value of $[OH]_i$. On the addition of bicarbonate a second penetrating molecule, CO_2, is formed, which for reasons discussed elsewhere (*J. Cell. Comp. Physiol.*, **7**, 351, 1936) leads to the accumulation within the cell of NH_4HCO_3 at a higher concentration than outside. An exchange of HCO_3 for Cl completes the entrance of NH_4Cl, the bicarbonate again being available to repeat the cycle. Because of the more favorable value of $[HCO_3]_i[Cl]_o - [HCO_3]_o[Cl]_i$ the entrance of Cl under these conditions is far more rapid than before. In agreement with the theory, two parts of the swelling curve of the erythrocyte may be distinguished, the first, which involves only undissociated molecules, being little affected by butyl alcohol, the second, which depends on an ionic exchange, being strongly retarded. Even without alcohol two parts of the curve are apparent if cyanide or sulphide be substituted for bicarbonate. The ineffectiveness of acetates etc. seems to be due both to a slower entrance of free acid and to a less ready ionic exchange. The addition of bicarbonate to ammonium citrate gives only the first part of the swelling curve, since there is no penetrating anion externally for which HCO_3 can be exchanged. For reasons discussed previously (*loc. cit.*) even the initial swelling is absent when borates are used.

Oxygen consumption and cell division of fertilized Arbacia eggs in the presence of respiratory inhibitors. M. E. Krahl, A. K. Keltch, and G. H. A. Clowes.

For fertilized eggs of *Arbacia punctulata* initially exposed to the reagents at 30 minutes after fertilization at 20° C., the levels of oxygen consumption prevailing in the minimum concentrations of reagent producing complete cleavage block were: In 0.4 per cent oxygen—99.6 per cent nitrogen, 32 per cent of control oxygen consumption; in 0.7 per cent oxygen—99.3 per cent carbon monoxide, 32 per cent of control oxygen consumption; in 1.6×10^{-4} M KCN, 34 per cent of control oxygen consumption; in KCN at 24° C. the value was 16 per cent of the control oxygen consumption.

The carbon monoxide inhibition of oxygen consumption and cleavage was reversed by light from a powerful carbon arc lamp. The percentage of inhibition of

oxygen consumption by CO in the dark is described by the equation $\frac{n}{1-n} \cdot \frac{pco}{po_2} = K$ where n is the fraction of oxygen consumption not inhibited, pco and po_2 the partial pressures of CO and O_2, respectively, and $K = 60$. A 20 per cent stimulation of oxygen consumption occurred in 10 per cent oxygen—90 per cent CO.

Spectroscopic examination of fertilized and unfertilized *Arbacia* eggs reduced by hydrosulfite revealed no cytochrome bands, although a band at 600–605 mμ corresponding to cytochrome *a* was found in *Arbacia* sperm. The thickness and density of the egg suspension used was such as to indicate that, if cytochrome is present at all, the amount in *Arbacia* eggs is extremely small as compared to that in other tissues having a comparable rate of oxygen consumption.

Three reagents poisoning copper catalyses, potassium dithiooxalate (10^{-3} M), diphenylthiocarbazone (10^{-4} M), and isonitrosoacetophenone (2×10^{-3} M) produced no inhibition of division of fertilized *Arbacia* eggs.

These results indicate that the respiratory processes required to support division in the *Arbacia* egg may be of a type not dependent on cytochrome for intermediate hydrogen transport, not dependent on a copper containing catalyst, and perhaps different in several essential steps from the principal respiratory processes of yeast or mammalian muscle.

Some factors affecting the rate of hemolysis of the mammalian erythrocyte by n-butyl alcohol. M. G. Netsky and M. H. Jacobs.

The hemolytic effect of solutions of *n*-butyl alcohol in isotonic NaCl varies greatly with small changes in concentration of alcohol and temperature. In certain parts of the range, concentration differences of 0.1 per cent or 0.0025 M butyl alcohol, and temperature differences of 0.25° C. are readily detectable. For the erythrocytes of eight species of mammals a maximum resistance to butyl alcohol hemolysis was found at pH 6.7–6.8, with times of hemolysis lower in the acid than in the alkaline range. The pH of the maximum lies near the isoelectric point of hemoglobin. If it be assumed that the alcohol causes a condition of cation-permeability of the erythrocyte, an effect for which there is already some independent evidence, the Gibbs-Donnan equilibrium demands a swelling of the cell on both sides of the isoelectric point; this might be expected to hasten the rate of hemolysis. Optical studies of volume changes made by the method of A. K. Parpart show that at a pH value of 8.60 hemolytic concentrations of butyl alcohol convert the usual shrinkage of the cells in alkaline solutions into a rapid swelling as demanded by the theory; swelling also occurs rapidly at pH 5.18, but very slowly at pH 6.70. Despite the similarity of the pH effect in different species, marked and characteristic specific differences were found in the absolute times of hemolysis. At the pH maximum, the order of resistance was:

monkey > man > dog > cat > rat > rabbit > beef > pig.

In accordance with the Gibbs-Donnan principle even low concentrations of non-penetrating non-electrolytes were found to cause a considerable retardation of alcohol hemolysis, but the effect proved to be more complex than a simple osmotic phenomenon, since with equimolecular concentrations of different non-electrolytes it was generally greater the greater the molecular weight of the added substance. This relation is seen in the following series for beef cells:

lactose > sucrose > mannitol > dextrose, levulose > xylose
> pentaerythritol, erythritol > malonamide.

Studies on the permeability-decreasing effect of alcohols and pharmacologically related compounds on the human erythrocyte. J. B. S. Campbell and M. H. Jacobs.

In extending the work previously reported by Jacobs and Parpart (*Biol. Bull.*, **73**, 380, 1937), it was found that the hemolysis of human erythrocytes in 0.3 M solutions of glycerol at pH 7.4 is characteristically retarded by methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl, *n*-hexyl and *n*-octyl alcohols, as well as by ethyl ether, chloroform, ethyl and phenyl urethanes and several other substances in a manner that in general runs parallel with their pharmacological activity. On reducing the pH below 6.0, substances of this type were found to give an acceleration rather than a retardation of hemolysis, as they do likewise with beef erythrocytes at all pH values studied. While the absolute time of hemolysis in 0.3 M glycerol solutions is more than doubled by lowering the temperature from 35° C. to 5° C., the minimal effective concentration of alcohol remains approximately constant over this temperature range. At body temperature, quantitatively measurable effects were obtained with ethyl ether, chloroform, and ethyl alcohol at concentrations considerably lower than those reported in the literature to exist in human blood in anesthesia and in alcoholic intoxication.

Quantitative studies of the rate of passage of protein and other nitrogenous substances through the walls of growing and of differentiated mammalian blood capillaries. Richard G. Abell.[1]

The rate of passage of nitrogenous substances through the walls of growing blood capillaries was observed with a transparent chamber, inserted in the rabbit's ear, called the "moat" chamber. In this chamber the growing capillaries can be seen with the high powers of the microscope, their condition recorded, and their area calculated. After passing through the walls of these capillaries, nitrogenous substances diffuse into a moat, or reservoir, of known volume, from which they can be removed and analyzed quantitatively.

(1) Analyses were made of the total nitrogen entering the moat during the first 24 hours following the introduction into the moat of a mammalian Ringer's solution. (2) The total surface of the capillaries involved was obtained from measurements of length and diameter. From these two sets of data the calculated amounts of total nitrogen passing through per sq. mm. of endothelial surface per 24 hours were, in 6 different chambers, as follows: (1) 0.091 mg.; (2) 0.113 mg.; (3) 0.102 mg.; (4) 0.097 mg.; (5) 0.046 mg.; (6) 0.081 mg.

The slower rate of passage of these substances (through the walls of the capillaries) in chambers 5 and 6 was associated with a slower rate of circulation in these two chambers, as observed with the microscope.

When the Ringer's solution was left in the moat for intervals of time longer than 24 hours, the total nitrogen content of the moat rose above the total non-protein nitrogen level of rabbit's blood within 5 days, which would seem to indicate that plasma protein passes through the walls of growing blood capillaries.

Analyses of the moat content for protein showed that this is the case. Using calculations similar to those described above in the one chamber containing blood capillaries in which such studies have so far been made (chamber 3), but basing the estimation on a collection period 48 hours in length, *protein* nitrogen came through the capillary wall at the rate of 0.039 mg. per sq. mm. of capillary surface per 24 hours. This compares with the figure for *total* nitrogen of 0.102 mg. Thus, of the *total* nitrogen that came through the walls of the growing capillaries in this chamber per 24 hours, approximately ⅓ was *protein* nitrogen.

The results secured with a new type of chamber, the "filter disc" chamber,

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indicate that mature blood capillaries are permeable to protein, but less so than are growing blood capillaries.

AUGUST 30

The occurrence of cytochrome and other hemochromogens in certain marine forms. Eric G. Ball and Bettina Meyerhof.

The process of oxygen utilization in mammalian tissue appears to proceed through a chain of iron porphyrin compounds composed of hemoglobin, myoglobin, cytochrome oxidase and the three cytochromes *a*, *b*, and *c*. Certain marine animals, however, possess instead of hemoglobin a copper blood pigment, hemocyanin, which functions in a manner similar to hemoglobin. The question, therefore, arises as to whether these organisms are also deficient in those other iron porphyrin compounds that compose the respiratory chain in mammalian tissue. We have therefore examined the following hemocyanin-containing animals for these compounds: *Venus mercenaria*, *Busycon canaliculatum*, *Limulus polyphemus*, *Homarus americanus*, and *Loligo pealeii*. The heart and some body muscles of all these organisms were found to possess cytochrome oxidase and the three cytochrome components. In addition we have tested extracts of these same tissues for succinic dehydrogenase and have found that it is present in amounts which parallel roughly the concentration of cytochrome in these same tissues. Myoglobin has been found only in *Venus mercenaria* and *Busycon canaliculatum*. The radula muscles of the latter are extremely rich in this iron compound.

Two additional hemochromogens have been observed in *Limulus polyphemus*. One is present in the abundant clot obtained from the blood of the animal. Its reduced form possesses an absorption band centered at λ560 mμ. The oxidized form shows no characteristic band. The other is present in the eggs. Its reduced form shows an absorption band centered at λ625 mμ while the oxidized form has a band centered at λ570 mμ.

It would therefore appear that the process of oxygen utilization in these organisms whose blood pigment is a copper compound is similar to that in mammals except for the substitution of hemocyanin for hemoglobin. This substitution can therefore not be ascribed to the inability of these animals to utilize iron or to synthesize the porphyrin prosthetic group characteristic of the iron respiratory pigments.

The eggs and sperm of *Arbacia punctulata* were also examined. The sperm were found to contain abundant cytochrome oxidase, cytochromes *a*, *b*, and *c*, and succinic dehydrogenase. Tests for these same compounds in the eggs were negative. Upon addition of pyridine and sodium hydrosulfite to a ground egg suspension a strong hemochromogen band centered at λ560 mμ appeared indicating that a hemin is, however, also a constituent of the eggs.

Some observations on cholinesterase in invertebrates. Carl C. Smith and David Glick.

A study of the distribution of cholinesterase in some invertebrate hearts and in various tissues of *Limulus polyphemus* was made in an attempt to find some basis for explaining certain observations previously made concerning their reaction to cholinergic drugs.

The manometric method of Ammon utilizing the Warburg apparatus was used. In the following table the activities found are expressed in cubic millimeters of carbon dioxide produced per fifty milligrams of tissue per thirty minutes.

Invertebrate Hearts	
Modiolus demissus	304
Callinectes sapidus	51
Limulus polyphemus	50
Pagurus longicarpus	49
Libinia dubia	37
Venus mercenaria	5.5

Tissues of Limulus polyphemus	
Cardiac nerve	446
Ventral nerve	216
Heart (segments 5–6)	50
Heart (segments 1–2)	34
Blood serum	42
Blood cells (clot)	33
Intestine	18
Skeletal muscle	4.5

The concentration of cholinesterase found seems to roughly follow the amount of nervous tissue present. The enzyme was found in the blood and tissues of arthropods and crustacea in which it has previously been reported absent. The insensitivity of the clam heart to eserinization can be explained on the basis of the low esterase content found.

Crystalline myogen. Kenneth Bailey.

The albumin fraction of rabbit skeletal muscle is obtained in the form of long thin needles in the following way: the perfused minced muscle is treated with an equal volume of ice cold 1 per cent NaCl, and after standing for one hour is pressed dry. The globulin fractions in the press juice are separated by addition of solid ammonium sulphate until the specific gravity of the solution at 25° reaches 1.175, the pH being maintained at 6.3. After filtration the liquid is acidified with dilute H_2SO_4 to a pH of 5.8 and after standing overnight is again filtered. More ammonium sulphate is fed in with slow stirring through a collodion membrane, the crystals separating when the specific gravity reaches 1.18; at a specific gravity of 1.21 crystallisation is complete.

Effect of increased intracellular pH on the physiological action of substituted phenols. J. O. Hutchens and M. E. Krahl.

1. The effects of five substituted phenols (2,4-dinitrophenol, 4,6-dinitro-o-cresol, 2,4,5-trichlorophenol, 2,4-dichlorophenol, and m-nitrophenol) on the respiration and cell division of fertilized eggs of *Arbacia punctulata* have been determined at an extracellular pH of 8.0 in the presence and absence of a concentration of ammonia (0.004 M NH_4Cl) sufficient to increase the normal cytoplasmic pH from the normal value of 6.8 ± 0.2 to approximately 7.2 ± 0.2.

2. The following results were obtained:

a. The relative and absolute stimulation of oxygen consumption produced by suboptimum concentrations of each substituted phenol was greater in the presence than in the absence of ammonia.

b. The relative inhibition of cell division by each concentration of each substituted phenol was the same in the presence and absence of the ammonia.

c. The optimum respiratory stimulating concentration for each substituted phenol was the same in the presence and absence of the ammonia.

3. These results confirm and extend the experiments of Krahl and Clowes [*J. Cell. and Comp. Physiol.*, **11**, 1 (1938)] in which the cytoplasmic pH was decreased by means of carbon dioxide. Both the present and previous series of experiments, so far as the experimental and theoretical limitations of the method permit, indicate that the substituted phenol anion is the intracellular active form for respiratory stimulation and that the substituted phenol molecule is the intracellular active form for inhibition of cell division. The experimental data are completely inconsistent with the suggestion, advanced by Tyler and Horowitz [*Biol. Bull.*, **75**: 209 (1938)], that the substituted phenol anion is the intracellular active form for inhibition of cell division.

Fatty acid compounds in the unfertilized egg of Arbacia punctulata. Albert E. Navez.

In a note in this *Bulletin* (1938) [1] it was pointed out that some "oil" was reacting with the Nadi reagent (used in experiments on indophenoloxidase) concurrently with its reaction with the oxidase. Variable results in the saponification value and in the iodine number of this "crude oil" raised doubts on the adequacy of ether as an extraction fluid. Even carbon tetrachloride extractions showed differences unless the solvent was specially purified.

In the present experiments cyclohexane and CCl_4 were used as extraction solvents; in some cases after treatment of eggs (packed solidly by centrifuging) with alcohol, in others in an atmosphere of nitrogen.

In the "crude oil" sterols and phospholipids are present (cf. also Mathews, 1913;[2] Page, 1927[3]). This oil, dark red in color, heavy in consistency, with a strong fish oil odor and slowly semi-drying in thin films, was fractionated after saponification.

The fatty acids separated are: saturated fatty acids, unsaturated fatty acids and fatty oxyacids. Their relative quantities seem to be variable with successive batches of eggs and moment in the season. The largest portion of fatty acids are unsaturated; we have isolated by Br derivatives small quantities of the diethylenic, larger amounts of the triethylenic and definitely indications of tetraethylenic (clupanodonic?) in small quantities.

The red color of the oil is interesting as it can be removed by adsorption on norite but with concomittant removal of some fatty compound. By successive acid and alkaline treatments the red color can be eluted from the adsorbing agent; it gives the absorption spectrum of echinochrome in CCl_4. The possibility is seen of the presence of a fatty acid derivative of echinochrome, playing a possible rôle on oxidations in the egg, in view of the ease of oxidation of the unsaturated compounds.

The unsaponifiable as yielded—unfortunately in very small quantities—small crystals on treatment by HCl gas in anhydrous acetonic solution, which might point to the presence of some unsaturated hydrocarbon (squalene). No detailed work done due to small quantity available. From the present observations it appears that the composition of the "crude oil" is not constant but varies with time, state of animals, feeding, method and length of keeping, perhaps temperature of sea water. No correlation has been found. Work on a larger scale is planned for the future.

Color changes in luciferin solutions. Aurin M. Chase.

During the spontaneous non-luminescent oxidation of *Cypridina luciferin*, partially purified by Anderson's method, the visible absorption spectrum of the solution, which has initially a slightly increasing absorption toward the shorter wavelengths, rises, producing a maximum at about 470 mμ, and then subsequently falls. This change is much more rapid and its magnitude greater in aqueous solutions of luciferin than in butyl alcohol solutions and the loss of luciferin (as measured by light emission) in aqueous solutions and butyl alcohol parallels the color change.

Hydrogen peroxide causes very rapid and almost complete loss of color in luciferin solutions, together with very rapid oxidation of luciferin.

Measured at pH's from 5.1 to 10.2 the change in the absorption spectrum is much faster at alkaline than at acid pH's and so is the decrease in concentration of luciferin under these conditions, using the luminescent reaction as a measure of luciferin concentration.

These facts indicate that the color changes observed represent changes in luciferin itself.

[1] Navez, A. E., 1938. *Biol. Bull.*, **75**: 357.
[2] Mathews, A. P., 1913. *Jour. Biol. Chem.*, **14**: 465.
[3] Page, I. H., 1927. *Biol. Bull.*, **52**: 164.

Anderson has demonstrated that when luciferase is added to a partially oxidized luciferin solution approximately the same amount of light is emitted, but much more slowly, as is emitted when luciferase is added immediately upon dissolving the luciferin. To explain this he has postulated that the non-luminescent oxidation of luciferin is reversible and, in the presence of luciferase, the slowly emitted light in the former case is controlled by the reduction of this reversibly oxidized luciferin. This reversible oxidation is believed to be represented by the initial rise in the absorption spectrum of luciferin solutions during non-luminescent oxidation in air. The subsequent decrease in absorption must indicate another reaction, possibly also an oxidation; probably irreversible.

A change in the absorption spectrum of luciferin solutions also occurs as a result of the luminescent reaction itself, and this color change may perhaps be used in studying the luminescent reaction.

Photodynamic action in the eggs of Nereis limbata. Fred W. Alsup.

Photodynamic effects can be produced in the eggs of *Nereis* by exposing them in solutions of rose bengal or eosin of the proper concentrations to sunlight or to light from a 1000-watt electric bulb. The effects consist of: (1) elevation of wide membranes, (2) germinal vesicle breakdown and (3) cytolysis of the eggs. The percentage of nuclear breakdown varies with exposure time and the concentration of dye. Solutions of rose bengal stronger than 1 part to 20,000 parts of sea water produce effects on the eggs in the dark. These dark reactions involve nuclear breakdown, staining of the entire eggs and cytolysis. With eosin a much stronger concentration is required to produce the same degree of reaction. Previously irradiated solutions of rose bengal produce no observable effects on the eggs, but when eggs are put into previously exposed weak solutions of the dye and then removed to sea water and fertilized, many cleave irregularly or cytolyze, indicating that the previously exposed solutions did have some effect on the eggs. Most eggs develop normally in these weak solutions, if the solutions have not been exposed to light. Solutions of eosin previously exposed to light produce nuclear breakdown in the eggs. No photodynamic changes can be produced in the eggs in the absence of free oxygen. KCN increases photodynamic action on the eggs as shown by increased percentages of nuclear breakdown. Relatively concentrated solutions of rose bengal bleached by sunlight have little or no effect on the eggs, since eggs can be fertilized and will develop normally in such bleached solutions, whereas they can not develop in unbleached solutions of the same concentration.

The same general effects obtained with the eggs of *Nereis limbata* were obtained with the eggs of *Arbacia punctulata.*

Cleavage delay in Arbacia punctulata eggs irradiated while closely packed in capillary tubes. Irving Cohen.

From the work of Henshaw and others it is known that X-rays administered to *Arbacia punctulata* eggs prior to fertilization cause a delay in the occurrence of first cleavage.

Doctor Failla suggested to the writer the problem of comparing the radiosensitivity of these eggs when irradiated in the ordinary way and closely packed in capillary tubes.

The general technique worked out by Henshaw, (*Am. Jour. Roentgenol. and Rad. Ther.*, 27, No. 6, June, 1932) has been followed in these experiments. Individual controls were set up for the two parallel series of experiments.

The results show that with equal dosages of radiation considerably less delay in cleavage is produced in the eggs irradiated in the capillary tubes. Doctor Failla predicted the result on the basis of his theory of the biological action of ionizing radiations (*Occ. Publ. Am. Ass. Adv. Sci.* No. 4, June, 1937). He has suggested that

owing to the greater complexity of the molecules within the egg, the increase in ion concentration resulting from the X-rays would be relatively greater in the cytoplasm than in the sea water. Therefore the radiation would cause an initial ionic unbalance across the cell boundary and this is assumed to enhance the radiation effect. On the other hand, when the eggs are closely packed in capillary tubes there is practically no sea water around the cells and the ionic unbalance should be much less. Accordingly the radiation effect should be much less marked.

It should be noted that while the experimental results confirm Failla's prediction it does not follow that the suggested explanation is necessarily correct. (This point is fully appreciated by Failla who has followed the work with interest.) There are, of course, other possible explanations.

The X-ray effect on the cleavage time of Arbacia eggs in the absence of oxygen. Rubert S. Anderson.

From a chemical viewpoint it is possible to consider that one result of the ionization produced by the absorption of X-rays is the formation of products having oxidizing and reducing tendencies. Experimentally, a number of authors have found that oxidation or reduction is one of the types of reactions produced by X-rays in simple chemical systems. They are especially common in the dilute aqueous solutions studied by Fricke and Clarke where most of the reactions occur indirectly through the water molecules.

This same type of reaction almost certainly occurs within living cells during irradiation and it is important to know if it plays any significant part in biological effects. Skoog concluded that this was true for certain plants and Fricke has suggested similar possibilities for some genic effects in *Drosophila*.

In the present preliminary experiments the primary object has been to see if the X-ray effect could be modified experimentally, as a first step toward finding out what reactions are important. *Arbacia* eggs have been used. Henshaw showed that irradiation of the unfertilized egg increased the time from fertilization to the first cleavage. If oxidations or reductions produced by the X-rays are an important factor in this delay it seemed possible that irradiation in the absence of oxygen would modify the effect.

Oxygen was removed from suspensions of eggs in Thunberg tubes by washing out with purified hydrogen. These eggs and control eggs were irradiated with 15,000 r.

All of the eggs were fertilized in air within four minutes after the end of irradiation and the time until 50 per cent of the eggs had cleaved was determined.

In all of fifteen lots of eggs the delay in cleavage was greater for the eggs irradiated in air than for those irradiated in hydrogen and this increase in delay averaged about 50 per cent. However, the variability was very large and ranged from a low of 13 per cent, which is probably not outside the error, to over 100 per cent for eggs from different females.

The absence of oxygen (or conceivably the presence of hydrogen) therefore does modify this X-ray effect in most *Arbacia* eggs. This is not presented as an argument for the importance of oxidations or reductions but these results do encourage investigation in that direction.

Fixation of X-ray effect by fertilization in Arbacia eggs. P. S. Henshaw.

We have shown previously that exposure of *Arbacia punctulata* eggs to X-rays causes a delay in the occurrence of the first cleavage, and further, that if an interval of time is allowed between the end of treatment and the moment of insemination, the effect is reduced or lost as a function of time—the latter being a change referred to as *recovery*.

While these findings were satisfactory in demonstrating that recovery takes

place so far as the first cleavage is concerned, they gave no information as to whether it is significant for subsequent cleavages and later development or whether it continues after fertilization. It is the purpose of this report to deal with these points.

The procedure followed was to treat a collection of eggs giving all the same exposure, fertilize samples of these at various times up to three hours after treatment, allow to develop for 48 hours, and then to classify the embryos as to degree of development. By the method used, it was possible to determine whether all samples had developed to the same extent and to compare the development in samples which had had different amounts of time for recovery to occur.

It was found first that development had proceeded farther in those samples which had been allowed the most time for recovery before fertilization; and secondly, that curves arranged to show the amount of development as a function of time, bore characteristic similarities to those based on delay in the occurrence of the first cleavage.

On the basis of these observations, therefore, it appears that the recovery from X-ray effect, which takes place before fertilization and is manifest by the time of the first cleavage, is significant in subsequent cleavage and later development; and consequently, that fertilization acts to fix whatever X-ray effect is present at the moment of fertilization.

Does the action of X-rays on the nucleus depend upon the cytoplasm? William R. Duryee.

Germinal vesicles from small ovarian eggs of three local species of frogs were irradiated both in intact eggs and in the isolated condition in Ca-free Ringer. Dosages varied from 500 to 50,000 r.[1] Chromosomes in nuclei isolated after previous irradiation *in situ* showed progressive injuries starting from 1000 r, in contrast to those in isolated irradiated nuclei, which even after 50,000 r showed no marked differences from the controls. No appreciable latent period in any of the 28 experiments was observed. In support of the conclusion that radiation damage to the chromosomes results primarily from chemical products of the injured cytoplasm is the fact that nuclei, having first been isolated and then placed in a concentrated egg brei and exposed to 50,000 r, showed typical chromosome defects of nuclei irradiated *in situ*.

Chromosome injuries were of three types: progressive loss of side branches or chromomere loops (which I described here last summer), fragmentation of the longitudinal chromonemata, and frequent separation of the members of synaptic pairs. Contraction of the chromosomes occurred when they were exposed either to irradiated or to non-irradiated injured cytoplasm as previously described under the term *Autofixation*. These changes are distinct from simple displacements of the chromosome pairs from their normal central positions in the nuclear matrix which depend on other factors.

Papers Read by Title

Moulting and viability after removal of the eyestalks in Uca pugilator. R. K. Abramowitz and A. A. Abramowitz.

Operative mortality following eyestalk removal in *Uca* is about 8 per cent for the first 24 hours. Eighty animals were isolated in paper cups (to abolish cannabalism) on the second day following eyestalk extirpation, and 25 normal animals were kept as controls under identical conditions. After one month, 12 per cent of the operated animals died directly without moulting, whereas none of the normal animals died without moulting. Moulting in the operated animals began on the tenth day, the percentage increasing rapidly as a hyperbolic function of time. Fifty per cent of the

[1] Irradiation experiments carried out by E. LP. ittle.

animals had moulted by the sixteenth day, and 96 per cent by the twenty-eighth day. Only 5 of the normal animals had moulted by the end of a month beginning the twenty-fifth day. The operated animals experienced considerable difficulty in moulting, many of them dying during or shortly after moulting. The total mortality was 74 per cent at the end of the month, 62 per cent being due to death in moulting. Forty per cent of the normal animals that moulted died during moulting. Blinded animals appear to lose pigment, an effect which becomes especially evident after moulting.

A new method for the assay of intermedin. A. A. Abramowitz.

The proposed method is based on previous observations that a pale denervated caudal band cannot darken during black-adaptation in hypophysectomized *Fundulus*, and that maximal sub-lethal doses of purified intermedin evoke a darkening in the denervated band but nowhere else in the integument of white-adapted fishes. In the caudal fins of 400 *Fundulus*, a 2 mm. band was made and the fishes white-adapted for 5 days, at which time both innervated and denervated regions of the tail were uniformly pale. In one series, graded doses of purified intermedin and in another, weighed samples of commercial pituitary powder (sheep whole gland) emulsified in distilled water were injected intraperitoneally into 20 fishes for each dose. The percentage of animals which responded was determined after a half-hour. The points fall on a smooth hyperbolic curve whose steepest part lies between 0 and 40 per cent. A unit of activity is defined as that amount of intermedin which darkens the denervated band in 25 per cent of the animals, at least 20 animals being injected. This test, in addition to being quantitative, seems to be quite specific. Twenty drugs, mainly alkaloids, were tested in various dosages, and all were ineffective in producing this reaction. In fact, the drugs usually darken the entire integument, leaving the pale band unaffected—an effect diammetrically opposite to that of pituitary intermedin.

Analysis of the electrical discharge from the cardiac ganglion of Limulus. Florence Armstrong, Mary Maxfield, C. Ladd Prosser, and Gordon Schoepfle.

The median ganglion of the *Limulus* heart contains two types of nerve cell, large unipolar ganglion cells found in segments 4 to 7 and small multipolar cells found in the outer portion of the whole nerve cord (Heinbecker, A. J. P., 1933, 1936). In action potential records of the activity of isolated portions of this ganglion we find no spontaneous discharge from segments 1, 2, 3, and 8. Rhythmic cardiac discharges occur in the intermediate segments. Occasionally, particularly in segments 5 to 7, there is a continuous spontaneous background upon which the rhythmic cardiac bursts are superimposed. Low potassium tends to favor the asynchronous background activity.

It has been postulated (Heinbecker et al.) that the small neurones are activated by the large pacemaker cells. A 0.1 per cent solution of nicotine, which abolishes all reflex activity in the central nervous system of the animal, was applied to the cardiac ganglion. The duration of the bursts remained constant, the interval between bursts diminished, thus increasing the cardiac frequency. This result indicates that activation of small neurones by the large ones is not by way of synapses.

Analysis of the activity in ganglia dissected down to a very few fibers showed that some neurones discharge only once per heart beat. Others discharge many times at a declining frequency during the burst. The cells providing the spontaneous background fire at a relatively constant frequency.

The intra-cellular distribution of reducing systems in the Arbacia egg. Robert Ballentine.

A comparison of the distribution of reducing activity (the dehydrogenase systems) with the cellular constituents has been studied by the manometric method of Quastel and Wheatley (1938) in centrifugally fragmented and whole *Arbacia* eggs. Allowing for considerable individual variation from urchin to urchin, it may be stated that the sum of the activities of the two halves is greater than that of the unfertilized egg, and approximately equivalent to that of the intact fertilized egg. Centrifugal and osmotic stretching of the egg, provided it is sub-threshold for activation, has little or no effect on the dehydrogenase systems. Since sedimentation of granules similar to that obtained in the half eggs is present in the centrifugally stretched cells, one is led to the conclusion that as far as the dehydrogenases are concerned, the process of fragmentation is equivalent to activation, although the halves are not induced to parthenogenetic development nor do they develop membranes. The exposure to 0.95 M sucrose, as employed in fragmentation, is without effect. On the basis of equivalent volumes, the red half has a greater activity than the white half, thus definitely indicating that the dehydrogenating systems are not exclusively limited to the clear cytoplasm, as is the peptidase activity (Holter, 1936). Rather it leads to the conclusion that a considerable portion of the substrate activation occurs at heterogeneous phase boundaries between the granules and the clear cytoplasm, or perhaps within the granular material itself.

Some effects of colchicine upon the first division of the eggs of Arbacia punctulata. H. W. Beams and T. C. Evans.

At room temperature 0.0002 molar concentration of colchicine in sea water inhibits cleavage of *Arbacia* eggs when applied at any time before (approximately) ten minutes of the appearance of the first cleavage furrows. Eggs left in this solution for one hour, washed and let stand in sea water show recovery as indicated by the appearance of cleavage furrows, many of which are abnormal. Eggs which have started to cleave when put into the colchicine solution continue the process until the two blastomeres are formed. However, all further cleavages are suppressed unless the colchicine is removed.

Eggs placed in the colchicine solution ten minutes after fertilization and centrifuged ten minutes later show a more marked stratification than do controls centrifuged for the same time and speed. Likewise, they show more stratification than do controls of the same lot fertilized ten minutes later and which are in approximately the same stages of division as were the experimental eggs when placed in the colchicine solution.

These results indicate that colchicine in concentrations sufficient to block cleavage acts by lowering the viscosity; or by inhibiting the normal rise in viscosity (gelation) that is associated with the appearance of the mitotic apparatus of the cleavage process. This is further substantiated by the fact that a disintegration of the asters may be observed when the eggs are placed in the colchicine approximately ten minutes before the appearance of the cleavage furrows. The rays seem to fade out, leaving only clear and often irregular areas in the position formerly occupied by the asters.

Temporal summation in neuromuscular responses of the earthworm, Lumbricus terrestris. E. Frances Botsford.

Temporal summation has been demonstrated in vertebrate smooth muscle and in the muscle of crustaceans and coelenterates. The phenomenon in smooth muscle of vertebrates has been attributed to the spreading of a chemical mediator through the tissue so that with each additional stimulation more muscle fibers contract. This

study is to demonstrate temporal summation in the muscles of the body wall of the earthworm and the dependence of this summation upon a chemical mediator.

The earthworm was arranged for recording the contractions of the longitudinal muscles by a weak isometric lever. Stimulating the nerve cord at 8-second intervals with a tetanizing current of constant intensity and brief duration produced a facilitation of the successive responses, in some cases for as many as sixteen times. The dependence of the magnitude of the response upon the frequency of stimuli was demonstrated conclusively by varying the frequency by means of a vacuum tube stimulator. Furthermore, at constant frequency there was an increase in response with increase in duration of the stimulation. Thus it is evident that the strength of the response is dependent upon the number of stimuli and temporal summation is shown to be characteristic of the muscle responses of the earthworm.

Since this summation was also true of a dorsal muscle strip, the phenomenon is not dependent upon the nerve cord, but is produced either in the peripheral plexus or at the neuromotor junction.

The dependence of temporal summation upon the chemical mediator acetylcholine was demonstrated as follows:

(1) Eserine caused no response in an unstimulated dorsal muscle strip, but electrical stimulation of an eserinized muscle strip caused an increase in tension similar to that produced by acetylcholine.

(2) When the interval between stimulations was increased to 5 minutes there was no summation of the successive responses. But when eserine was applied to the muscle summation occurred in spite of the long interval between stimulations.

Since the muscle of the earthworm is very sensitive to acetylcholine, these experiments indicate that temporal summation is brought about by the spread of acetylcholine from the neuromotor junctions.

The source of chromatophorotropic hormones in crustacean eyestalks. F. A. Brown, Jr.

Through a series of injection experiments in which extracts of whole eyestalks and certain portions of eyestalk tissue were injected into *Palaemonetes* and *Uca* as test animals it has been demonstrated that the active source of chromatophorotropic hormones is a small, translucent, or bluish white mass located in the dorsal or dorsolateral region of the eyestalk. This tissue appears to be the sinusgland of Hanström and constitutes a definite gland which can dissected out easily in the forms investigated: *Cambarus, Carcinus, Callinectes, Libinia, Uca, Pagurus, Crago,* and *Palaemonetes.* In the last two named forms the gland is readily visible in the normal living animal. Quantitative studies of the effects of extract of the gland show that better than 80 per cent of the activity of the whole eyestalk extract is to be found in extracts of the minute gland. The difference of about 20 per cent can be accounted for by diffusion of substance from the gland into other eyestalk regions. Furthermore, the effect of the sinusgland by itself is qualitatively indistinguishable from that of the whole eyestalk of the same species judging by the relative effects of the sinusgland and whole eyestalk extracts upon the red chromatophores of *Palaemonetes* and the black ones of *Uca.* Implantation of the sinusgland of *Carcinus* into the abdomen of eyestalkless *Palaemonetes* has given confirmation of its chromatophorotropic activity as a single implant maintained the red pigment of *Palaemonetes* more or less concentrated over a five-day period at the end of which time the gland apparently became functionless.

Comparative effects of sinusgland extracts of different crustaceans on two chromatophore types. F. A. Brown, Jr. and H. H. Scudamore.

Extracts of the sinusglands of seven crustaceans (*Callinectes, Carcinus, Libinia, Pagurus, Uca, Crago,* and *Palaemonetes*) were each tested simultaneously on the red

chromatophores of eyestalkless *Palaemonetes* and the black chromatophores of eyestalkless *Uca*. The order of effectiveness of the different sinusgland extracts upon the two chromatophore types were not the same, some extracts having relatively more effect upon the *Uca* black and others upon the *Palaemonetes* red chromatophores. This was expressed in the form of a ratio:

$$\frac{\text{Effect upon } \textit{Uca} \text{ black chromatophores}}{\text{Effect upon } \textit{Palaemonetes} \text{ red chromatophores}}.$$

The values of this ratio when sinusglands alone were used showed a definite sequence which was substantially repeated when whole eyestalks were used. Of the crustaceans examined *Crago* showed the highest value for the ratio while *Palaemonetes* and *Callinectes* showed the lowest. To support an hypothesis that two hormonal substances are to be found in different proportions in the various sinusglands, the discovery was made that dried sinusglands, or eyestalks, extracted with absolute ethyl alcohol yielded a fraction with a very low value for the above-mentioned ratio while a sea-water extract of the alcohol-insoluble residue yielded a fraction with a very high value. Thus, there seems to have been effected a partial separation of two chromatophorotropic principles from the crustacean sinusgland.

On the control of the dark chromatophores of Crago telson and uropods. F. A. Brown, Jr. and H. E. Ederstrom.

Since Koller's work indicating the presence of a rostral organ secreting a pigment-dispersing hormone there has been general lack of confirmation and even denial of its actuality. An exhaustive series of injection experiments in which extracts of various regions and tissues of the body of *Crago* were injected into eyestalkless *Crago* has disclosed what appears to be the normal source of a dispersing humoral substance for the dark pigment of the telson and uropods. The only tissue of the many tried which yielded darkening of the tail in practically 100 per cent of the trials was the central third of the circum-oesophageal connectives including the connective ganglion and a short portion of the connectives immediately posterior to the ganglion. From eyestalkless and black-adapted animals this middle third was usually the only effective region but in the case of white-adapted animals frequently all three portions of the connectives (anterior, middle, and posterior), and often even the posterior portion of the brain were somewhat active. This last was probably due to the diffusion of substance through the connectives. An extract of *Palaemonetes* connectives acts similarly upon *Crago*, though extracts of connectives of *Carcinus*, *Uca*, *Libinia*, *Pagurus*, and *Callinectes* fail to produce the response. The dispersing action of the connective hormone is annulled by extracts of eyestalks of *Crago* or *Palaemonetes* but is apparently uninfluenced by eyestalk extracts of *Carcinus*, *Libinia*, or *Uca*. The latter extracts exert the interesting effect of blanching strongly the trunk and leaving the black "tail" more conspicuous than ever, by contrast. The observations thus indicate definitely that there is an extra-eyestalk origin of a chromatophorotropic hormone and that *Palaemonetes* and *Crago* have a principle in their eyestalks not to be found in the eyestalks of *Carcinus*, *Libinia*, and *Uca*.

Micromanipulation of salivary gland chromosomes. John B. Buck.

Micromanipulation of normal salivary gland chromosomes of *Chironomus plumosus, in vitro*, proved infeasible because dissection of the cells causes immediate and marked abnormalities in the chromosomes. However, immersion of the gland in a hanging drop of isotonic Ringer's over the vapor of osmium tetroxide for 15 to 18 hours at 5° C. renders the cytoplasm and nuclear membrane sufficiently brittle so that individual chromosomes can be isolated. These chromosomes resemble those *in vivo* very closely in regard to minute visible structure and dimensions, and in addition partly retain the power of living chromosomes of responding reversibly to

osmotic changes in their environment. The mechanism of action of the osmic vapor is obscure, but apparently involves a surface reaction, rather than impregnation, since the principal effect is loss of stickiness, and no osmium could be found inside the chromosomes following reduction.

The following results were obtained from manipulation of these chromosomes:

(1) A photographic record was obtained of the stage-by-stage reversible transformation of staggered transverse rows of achromatic droplets (honeycomb) into longitudinal parallel thread-like striations, supporting Metz's view that the latter are artifacts.

(2) Most of the longitudinal stretching occurs in the interband regions. Return to original length in relaxation may occur after up to 300 per cent stretch, and at least 500 per cent stretch may be sustained before breakage occurs. Breakage always occurs in the interband regions and in a straight line at right angles to the long axis of the chromosome.

(3) The somatically synapsed homologs are so intimately fused that forces sufficient to break the chromosome cannot separate them.

Effects of Roentgen radiation on certain phenomena related to cleavage in Arbacia eggs (Arbacia punctulata). T. C. Evans and H. W. Beams.

Fertilized eggs irradiated at 7,400 r/m. (minute exposures with a maximum dosage of six) showed subsequent delay in first cleavage which increased exponentially with the dosage.

Clumping of the eggs was noticed in the irradiated lots and it was found (by staining with Janus green) that the jelly, which surrounded the control eggs, was missing in the irradiated lots. Absence of the jelly was also noted in irradiated lots of unfertilized eggs. The effect was noticed immediately after irradiation and appears to be a direct radiation action as eggs placed in irradiated sea water were found to retain their enveloping jelly as long as did eggs in untreated water. Dosages below 10,000 r were not completely effective. Some fertilized eggs were supported in a hanging drop over polonium and were examined at the time when the controls were in the eight-cell stage. Some of the eggs in the drop were apparently not affected but some of them showed unilateral delay in cleavage. Such eggs stained with Janus green possessed the jelly only on the side of the unaffected blastomeres.

Irradiated sperm were found to lose their motility and fertility more rapidly than did the controls. Irradiated sperm (radiation sufficient to delay subsequent cleavage of eggs fertilized with treated sperm) were found to produce the initial rapid increase in oxygen consumption, as noted for controls, upon fertilization. Dead sperm, when added to the egg suspension in the respirometer, failed to produce the increased oxygen uptake. It appears that the rapid increase in oxygen consumption noted in normal fertilization may be related to the entrance of the sperm (or other related surface actions) regardless of the subsequent fate of the sperm nucleus.

Fertilized eggs irradiated as high as 37,000 r showed less stratification than controls when centrifuged at the same time.

The above findings are apparently unrelated in the present preliminary state of the investigation. It is evident, however, that radiation may produce several quite different immediate biological effects in the same cell and that such effects must be considered in attempting to formulate any possible fundamental biologic action of radiation.

Chemical and mechanical properties of two animal jellies. John D. Ferry.[1]

The jelly surrounding the eggs of *Arenicola cristata*, when dialyzed free of salts, contained only 0.2 per cent solid matter. It could be reversibly shrunk by concen-

[1] Society of Fellows, Harvard University.

trated salt solutions or by alcohol, acetone, or dioxane; it was not dissolved by zinc chloride, urea, or dilute acid. Qualitative tests showed that the material was a polysaccharide containing uronic acid. The jelly was precipitated by barium chloride (thereby releasing the eggs) in the form of long fibers, which swelled only slightly in cold dilute acid or salt solutions.

The relaxation of shearing stress in the dialyzed jelly, shrunk to different extents, was examined in a concentric cylinder apparatus. Analysis of the decay curve indicated two relaxation times of the order of 2 and 100 minutes respectively, which decreased somewhat with increase of temperature, but showed little change in a concentration range where the rigidity varied tenfold.

Fresh specimens of *Mnemiopsis leidyi*, drained of excess sea water, were treated with borate buffer at pH 9.5 to dissolve the softer parts, and the residue discarded. When the solution was brought to pH 3, a viscous gel formed, and separated as a gelatinous, stringy clot. This was compressed by centrifuging and extracted with alcohol, thereby removing a small quantity of yellow lipoid material. The clot was resuspended in water and washed free of alcohol.

Qualitative tests on the material thus purified showed it to be a mucoprotein, containing 12 per cent nitrogen, and small amounts of sulfate sulfur and cystine. It swelled increasingly with increasing pH, dissolving alkaline to pH 8. In the neighborhood of pH 8 it formed highly viscous gels; this property was, however, destroyed by concentrated urea or by boiling.

The relaxation of shearing stress in a 0.1 per cent solution in M/6 borate buffer at pH 8.8 showed a relaxation time of the order of 2 minutes, which decreased with increasing temperature.

Response of frog striated muscle to $CaCl_2$. Judith E. Graham and F. J. M. Sichel.

The local application of $CaCl_2$ to the surface of a length of isolated skeletal muscle fibre causes a marked reversible shortening of the muscle substance, as pointed out by Chambers and Hale and by Keil and Sichel. This behavior of the isolated length of fibre is in marked contrast to that of the intact muscle, where no such shortening occurs even with concentrations of $CaCl_2$ as high as 400 mg. per cent. Since KCl-treated muscles have been shown by one of us to resemble in some respects the isolated fibre preparation, it was thought advisable to investigate the possibility that such muscles might behave like the isolated fibre also with respect to $CaCl_2$.

The isolated fibre shows no marked response to the injection or local application of KCl. The intact muscle (frog) when placed in a modified Ringer's solution containing 400 mg. per cent KCl undergoes a rapid transient contracture which disappears in 3 to 5 minutes. Subsequent to this treatment of the muscle with the KCl, and its response, the muscle will shorten markedly if placed in a similar solution containing 400 mg. per cent $CaCl_2$. This shortening is reversibly maintained in the presence of the excess $CaCl_2$ if the excess KCl is still present in the solution, but subsides slowly if only the normal concentration of KCl is present. This sensitization of the muscle to $CaCl_2$ by excess KCl is reversible; that is, washing the muscle in Ringer's solution after the transient KCl contracture renders it insensitive again to the action of $CaCl_2$. No antagonism of the $CaCl_2$ contracture by an equal KCl concentration could be detected.

Curarized muscle in Ringer's solution, and muscle in isotonic sucrose behaves like the KCl-treated muscle.

The permeability of the toadfish liver to inulin. Charlotte Haywood.

An earlier investigation (Haywood and Höber, *Jour. Cell. Comp. Physiol.*, **10**, 305, 1937) has indicated that the relatively large, lipoid-insoluble inulin molecule penetrates the isolated bullfrog liver, from perfusion fluid to bile, as through a passive

filter. The present study demonstrates that in a living, unanaesthetized animal, retaining its normal blood supply to the liver, administered inulin can also enter the bile.

The toadfish was used because its aglomerular kidney fails to eliminate inulin. (Shannon, *Jour. Cell. Comp. Physiol.*, 5, 301, 1934); 1.5 to 2. grams inulin per kilogram of body weight were injected intramuscularly a day or more before collection of bile samples. During collection, fish were strapped down and kept alive by a stream of water entering the mouth and directed over the gills. A ventral incision was made, the bile duct ligated, the gall bladder drained and cleaned, and a cannula inserted, after which the incision was closed, leaving the cannula protruding. Such preparations secreted up to 3 or 4 mg. bile per hour per gram of liver over a collecting period of 11 to 21 hours, after which a blood sample was drawn, usually from a caudal vessel, occasionally from the heart. Survival of fish following the operation was obtainable, often for several days.

Twenty-fold dilutions of bile and of protein-free blood plasma were analyzed by the Shaffer-Somogyi method for reducing substance. The difference in reducing substance before and after hydrolysis with H_2SO_4 represents the amount of inulin hydrolyzed to levulose.

A series of nine experiments showed the concentration of inulin in the bile to range between 55 and 73 per cent of that in the blood plasma. These figures are corrected for a small amount of reducing substance found in the bile in control experiments, without inulin.

A further series to determine the effect of a choleretic is under investigation.

Activation of Cumingia and Arbacia eggs by bivalent cations. Josephine Hollingsworth.

Eggs of *Cumingia tellinoides* are activated by isotonic solutions of $SrCl_2$, $CaCl_2$ and $BaCl_2$. The degree of effectiveness of the various salts follows the order named. The various pHs from 6.1 to 8.6 are equally favorable for activation. The time of polar body formation in eggs activated by bivalent cations is approximately the same as the time of polar body formation in eggs activated by sperm. The addition of sea water or the addition of isotonic solutions of NaCl or KCl tends to inhibit activation by bivalent cations. This inhibiting effect increases as the proportion of sea water or the concentration of the monovalent cations increases.

Eggs of *Arbacia* are activated by isotonic solutions of $CaCl_2$, $SrCl_2$, $MgCl_2$ and $BaCl_2$. Calcium ions are more effective than strontium, magnesium and barium ions. The action of the last three cations is somewhat variable. Whereas the bivalent cations act rapidly on *Cumingia* eggs, *Arbacia* eggs must be exposed to them for hours before any effect is observed. In eggs exposed to calcium ions, there is a much higher percentage of cleavage in ovary eggs which have been washed than in ovary eggs which have not been washed; in shed eggs than in ovary eggs either washed or unwashed; and there is a slightly higher percentage of cleavage in shed eggs that have been washed than in shed eggs that have not been washed. The highest percentage of cleavage takes place at pH 9.0. Below pH 8.8 the percentage of cleavage is usually small and above pH 9.2 the percentage of cytolysis is large. Whereas the addition to the calcium solution of isotonic solutions of NaCl or KCl tends to inhibit the activation of *Cumingia* eggs, in certain proportions the addition of these solutions to the calcium solution may increase the percentage of activation of *Arbacia* eggs while in other proportions the addition of these solutions has the opposite effect.

The vacuole systems of a fresh water limacine Amoeba. Dwight L. Hopkins.

By means of high power apochromatic objectives and staining with Nile blue sulfate, neutral red and Janus green, each type of vacuole and granule found in this amoeba has been followed from its origin to its disappearance. In general there are two systems of vacuoles. In active feeding amoebae the vacuoles arise from the protoplasm. One set of vacuoles, by swelling and coalescence, form the "contractile vacuoles" which are periodically evacuated to the outside. A second set of vacuoles coalesce with engulfed food and form the food vacuoles which after digestion has taken place are evacuated to the outside. Evacuation of the food vacuoles generally is independent of the contractile vacuoles. Occasionally, however, a food vacuole may coalesce entirely with a contractile vacuole and then this resulting food-contractile vacuole is evacuated. Again, the fluid portion of the food may run into the contractile vacuole leaving the food residue practically in contact with the protoplasm. Following this the food residue is soon evacuated but independently of the contractile vacuole.

In feeding or slowly feeding amoebae granules stainable with neutral red, Janus green B, and Nile blue sulfate arise in the protoplasm instead of food vacuoles. Under certain conditions more abnormal than those which cause cessation of feeding the contractile vacuole system is retarded and granular structures stainable with Janus green B, but not with Nile blue or neutral red, replace the fluid vacuoles which form the contractile vacuoles. Under favorable conditions the contractile vacuole system is not conspicuously stained with Janus green B. At a certain intermediate stage granulation of the contractile vacuole system becomes stainable with Janus green, but still these Janus green stained vacuoles can be observed to coalesce, swell and form the contractile vacuole in which a greenish-blue tinge definitely can be detected.

Cytological studies on androgenetic embryos of Triturus viridescens which have ceased development. Cornelius T. Kaylor.

Fankhauser (*J. E. Z.*, **68**, 1934) has shown that there is a high death rate in developing egg fragments of the European newt, *Triton palmatus*, during blastula and gastrula stages and that this is caused by the presence of irregular numbers of chromosomes in the cells of the embryos. A correspondingly high mortality during these same stages of development was observed in my experiments on androgenesis in eggs of *Triturus viridescens* (*J. E. Z.*, **76**, 1937). It was, therefore, reasonable to expect that the same abnormal chromosomal conditions as were found in *Triton palmatus* egg fragments would be responsible for the death of these androgenetic embryos of *viridescens* during the blastula and gastrula stages.

In a study of about 65 blastulae and 8 irregular gastrulae fixed at cessation of development, it was found that the cells of all these embryos were equipped with subhaploid to superhaploid numbers of chromosomes. The cessation of development was substantiated by the onset of cytolysis in all these cases. In a preliminary study of chromosome numbers in more advanced embryos which have ceased development, that is, neurulae and tail bud stages, it was found that there were only four questionable counts in over 100 which showed other than the haploid number of chromosomes.

It appears, then, that at least the complete haploid set of chromosomes is necessary in androgenetic embryos of *Triturus viridescens*, the same as in merogonic *Triton palmatus* embryos, if they are to develop beyond gastrulation.

Also a large number of mitoses were found in androgenetic embryos of *T. viridescens* fixed while still developing, which had no chromosomes at all on the spindle. Apparently cell division can proceed in the *T. viridescens* egg as well as in *Triton palmatus*, in the absence of chromosomes.

Effect of leukotaxine on cellular permeability to water. Valy Menkin.

The writer has recently succeeded in demonstrating the presence of a crystalline nitrogenous substance from inflammatory exudates capable *per se* of increasing capillary permeability and of inducing the migration of polymorphonuclear leukocytes. The liberation of this substance offers a reasonable explanation for two of the basic sequences in the development of the inflammatory reaction (*J. Exper. Med.*, 1936, **64**, 485 and 1938, **67**, 129, 145). This substance has been named *leukotaxine.*

In an endeavor to determine the mechanism of action on individual cells, the effect of leukotaxine on the permeability of ova of *Arbacia punctulata* was studied. Ova were exposed for intervals varying from 20 minutes to about an hour and a half to concentrations of leukotaxine ranging from about 3 to 8 mg. per cc. The eggs were subsequently immersed in hypotonic sea water (50 per cent concentration) and their degree of swelling measured from minute to minute with an eyepiece micrometer. A large number of ova were thus studied for a total period of 6 to 8 minutes. Their mean diameter served as a basis for the calculation of cell volume as previously described in the various contributions of Lucké and McCutcheon (cf. *Physiol. Rev.*, 1932, **12**, 68). The permeability was also obtained from the equation utilized by these workers: Permeability $= dV/dt/S(P - Pex)$.

The duration of exposure to leukotaxine prior to immersion in the hypotonic medium was found to be relatively inconsequential provided the pH had previously been adjusted to approximate that of sea water. A failure to follow this precaution might ultimately induce a change in ova exposed for long intervals to an acid pH that tends to reduce their swelling capacity when placed in a hypotonic medium.

The results of several experiments on a considerable number of ova indicate that leukotaxine appreciably increases the permeability of *Arbacia* ova to water. The extent of augmented permeability over that found in the case of normal ova in a similarly hypotonic medium is about twofold.

Effect of leukotaxine on cell cleavage. Valy Menkin.

Leukotaxine induces increased capillary permeability and migration of polymorphonuclear leukocytes in mammalian tissue (Menkin, *Physiol. Rev.*, 1938, **18**, 366). Its effect on ova of *Arbacia punctulata* is to enhance further their permeability to water when immersed in a hypotonic medium.

Does leukotaxine induce sufficient injury to ova to influence cleavage development? A series of observations have yielded the following results, summarized in brief:

1. Leukotaxine-treated ova manifest the usual fertilization reaction when exposed to sperms. The fertilization membrane, however, appears as a distinctly narrower zone than is seen under normal circumstances.
2. An appreciable number of fertilized ova fail to segment when exposed, for even a few minutes prior to fertilization, to a solution of leukotaxine.
3. The rate of cleavage of leukotaxine-treated ova tends to be retarded.
4. In the leukotaxine-treated group a considerable number of dividing eggs reveal atypical forms exemplified by unequal cleavage.
5. Sperms immersed for about an hour in sea water containing leukotaxine fail to fertilize normal ova.

These results indicate that leukotaxine seems to be definitely injurious to isolated cells as exemplified in the ova and sperms of *Arbacia punctulata.* In the case of ova this is manifested by increased permeability to water and by an appreciable inhibition to normal cleavage.

Response of the Arbacia egg cortex to chemical and physical agents in the absence of oxygen. Floyd Moser and J. A. Kitching.

Previous attempts to determine whether membrane elevation can be initiated in the *Arbacia* egg in the absence of oxygen have failed because of the fact that the

sperm is immobilized under these conditions. Thus there is no proof that the *Arbacia* egg cortex could not respond in the complete absence of oxygen if it were given an adequate stimulus. In the present experiments mechanical and chemical agents have been used, to avoid the effect of oxygen lack upon the sperm.

Drops of *Arbacia* eggs and of the various agents required were suspended from a cover slip sealed with vaseline over a modified Englemann gas chamber. A monolayer of ferric stearate (see Ballentine, *Science*, **89**, 1939) was laid down on the cover slip to prevent coalescence when several drops were employed. A short, wide, bent, mercury-filled, glass side-arm allowed gas-tight manipulation of a microneedle or fine glass loop within the chamber. Oxygen-free hydrogen was passed through the chamber at 50–75 cc. per minute, and adequate time was allowed for equilibration. Mixing of the drop was achieved by slight shaking of the chamber, and transfer of the eggs with little fluid from one reagent to another was accomplished by means of the loop.

Eggs treated with saponin (1/4 of 1 per cent, in sea water), molar urea, and molar sucrose solutions in the absence of oxygen exhibited the same characteristic cortical response and membrane elevation as that obtained in air (see Moser, *J. E. Z.*, **80**, 1939). Moreover, the time relationships were much the same, regardless of whether the experiments were made in an atmosphere of hydrogen, carbon-dioxide-free air, or air. Shortly after the response to the non-electrolyte solutions in the absence of oxygen, the elevated membranes disappeared (see Moser, *Biol. Bull.*, **73**, 1937). Typically the response to saponin was followed some minutes later by cytolysis. The response to pricking with a microneedle under anoxic conditions was not unlike that obtained in air (see Moser, *J. E. Z.*, **80**, 1939).

Eggs transferred under anoxic conditions to hypertonic sea water exhibited cortical alveolar swelling (see Hunter, *J. C. C. P.*, **9**, 1936), but no membrane elevation. When, however, the eggs had previously been treated with urea, which itself initiates the cortical response, no such cortical alveolar swelling took place.

When, by means of the loop, eggs were transferred in the absence of oxygen first to urea, and then through several changes of sea water, they subsequently cleaved when exposed to air, but did not cleave when kept under anoxic conditions. Eggs left in urea undergo amoeboid movements in air (Moser, unpublished observations), but in the absence of oxygen no such movement took place. Stoppage of this movement in absence of oxygen was reversible.

Further studies on regeneration in Fundulus embryos. S. Milton Nabrit.

Due to the fact that time recorded in terms of days lapsed since fertilization is not an adequate criterion of developmental time for *Fundulus*, the results obtained from experimental development on this form cannot be readily compared. Some of the differences in results obtained after the removal of the distal end of the tail of the fish may be accounted for on that basis. Other differences, however, are not so readily explained.

The Nicholas [1] technique was employed to remove *Fundulus heteroclitus* embryos from their chorions six days after fertilization. At this time the embryos were fitted to the Oppenheimer [2] developmental schedule for normal development at 25° C. The embryos were between the stages 24–25, about 80 hours. The natatory fold was elevated from the distal end of the tail up to the third segment from the distal end. The rounding of the caudal fin had not begun. The operations for removal of the chorions and for the distal two segments of the tail were performed in amphibian Ringer's solution. After twelve hours some fish were transferred to sea water; after twenty-four hours some fish were placed in 4/5 Ringer's and 1/5 sea water by volume.

[1] Nicholas, J. S., 1927, *Proc. Nat. Acad. Sci.*, **13**.
[2] Oppenheimer, J., 1936, *J. E. Z.*, **73**.

About 20 per cent of the fish kept in the modified Ringer's solution and 10 per cent of those transferred to sea water differentiated tail fins without replacing the missing tail segments. New rays were first observed in those in the modified Ringer's solution in 7 to 9 days. New rays were observed in those in sea water in 11 to 16 days.

Birnie [3] reported that five-day-old fish cut in sea water or in isotonic sodium chloride solutions that afterwards were transferred to sea water did not differentiate tail fins in 65 days. I previously reported that cauterized tail stumps would differentiate new fins in fish that were 9 days old at the time of removal of the distal end.[4] Therefore, to assume that some of the fish in these experiments had not arrived at the critical period for setting the differentiation, and that most of them had, would necessitate the supposition that cautery causes a reversal or recovery of the capacity to differentiate the caudal fin. The other alternative appears to lie in the nature of the healing process. If sloughing occurs, the chances that the natatory fold will close over the cut stump in time to become infiltrated with mesenchyme are greatly diminished. In such cases rays may not differentiate. It is rather striking that if rays differentiate at all it is quite early. The fold is a regressive structure in *Fundulus* and appears to depend upon fin differentiation for persistence. I have not obtained caudal regeneration in the fry of *Fundulus*, although it has been reported in several other fishes.

The action of certain drugs on the intact heart of the compound ascidian, Perophora viridis. A. J. Waterman.

Previous work has been done on *Molgula*, *Ciona* and certain salps (Hunter, Schultze, Bacq, etc.). In *Perophora* the abvisceral beats (toward branchial basket) greatly outnumber the advisceral; both are highly variable.

Adrenalin, mecholyl and acetylcholine excite dominance of the advisceral center, but it is a question if they all act in the same way and on similar mechanisms. Adrenalin increases the number of abvisceral beats and the length of rest periods, decreases the advisceral beats and causes irregularity. In 1–125,000 dominance lasts a few minutes. In 1–20,000 the advisceral beats are suppressed for many hours when transferred during the abvisceral series; if during the advisceral, several reversals occur before the abvisceral become continuous ('death sign' of Schultze). After recovery in sea water or oxidation of adrenalin (*Perophora* accelerates the latter) these results are reproducible. Occasionally 1–100,000 increases the number proportionately in both directions. One to 15,000 inhibits dominance, and beating from both ends occurs without coordinating rhythm. Mecholyl (1–5,000 to 1–30,000) is less effective and abvisceral dominance lasts about 10–30 minutes. In acetylcholine the time varies from 4–9 minutes. Certain other effects are also different.

Atropine (1–2,500 to 1–5,000) causes irregularity, stops the heart in 5–30 minutes, and tends to reduce or abolish the exciting action of mecholyl; but the latter restores an atropine-poisoned heart to near normal. Acetylcholine and mecholyl influence the action of each other. Strychnine and colchicine are depressant without significant evidence of stimulation or of differential effect on the heart centers. Reaction and recovery occur quickly. No prolonged contraction of the animals occurred except with lethal concentrations of these drugs. Some of the observed effects differ from those described for other ascidians. In certain respects these results resemble those obtained with crustacean hearts.

[3] Birnie, J. H., 1934, *Biol. Bull.*, **66.**
[4] Nabrit, S. M., 1938, *Jour. Exper. Zool.*, **79.**

An effect of the injection of a solution of dihydroxyestrin into castrated female frogs, Rana pipiens. Opal Wolf.

In 1928 Wolf reported that subcutaneous injections over a long period of time, of a water-soluble extract prepared from mammalian ovaries prevented the involution of the oviducts of female frogs castrated in the autumn. Wolf also demonstrated in 1929 that implants of the anterior lobe of frog pituitary evoked the reproductive processes of both the male and female frogs, *Rana pipiens*, as far out of season as September.

Further studies in 1938 showed that the ovary and the oviduct of the frog during the summer months were enlarged following pituitary stimulation, the latter presumably as a result of an increased output of ovarian hormone.

The present study was undertaken to show the effect of injections of a solution in sesame oil of the pure crystals of dihydroxyestrin (a-estradiol benzoate).[1] Female frogs were castrated early in July when the oviducts are very small and were allowed to recover fully from the effects of the operation. Approximately five grams of lean beef were fed daily, the animals gained in weight and appeared in excellent condition. From August 10 to August 17 inclusive, a total of 1000 rat units per frog of the solution was injected into the thigh muscles. An average increase of 47.1 per cent in the weight of the oviducts in proportion to the body weight (more marked in diameter than in the length of the oviducts) of the injected animals over the castrate controls had occurred as a result of the eight days of injection.

[1] The hormone was furnished through the courtesy of Dr. Max Gilbert of the Schering Corporation.

I wish to thank Miss Naomi de Sola Pool for technical assistance.

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CONTENTS

Page

SOUTHWICK, WALTER E.

Activity-preventing and Egg-Sea-Water Neutralizing Substances from Spermatozoa of Echinometra subangularis 147

SOUTHWICK, WALTER E.

The "Agglutination" Phenomenon with Spermatozoa of Chiton tuberculatus 157

KANDA, SAKYO

The Luminescence of a Nemertean, Emplectonema kandai, Kato 166

FAWCETT, DON WAYNE

Absence of the Epithelial Hypophysis in a Fetal Dogfish Associated with Abnormalities of the Head and of Pigmentation 174

GOODRICH, H. B., AND PRISCILLA L. ANDERSON

Variations of Color Pattern in Hybrids of the Goldfish, Carassius auratus 184

GOODRICH, H. B., AND J. P. TRINKAUS

The Differential Effect of Radiations on Mendelian Phenotypes of the Goldfish, Carassius auratus 192

JOHNSON, W. H., AND J. E. G. RAYMONT

The Reactions of the Planktonic Copepod, Centropages typicus, to Light and Gravity 200

ROSE, S. MERYL

Embryonic Induction in the Ascidia 216

PORTER, K. R.

Androgenetic Development of the Egg of Rana pipiens 233

BUTCHER, EARL O.

The Illumination of the Eye Necessary for Different Melanophoric Responses of Fundulus heteroclitus 258

BRAGG, ARTHUR N.

Observations upon Amphibian Deutoplasm and its Relation to Embryonic and Early Larval Development 268

VON BRAND, THEODOR, NORRIS W. RAKESTRAW AND CHARLES E. RENN

Further Experiments on the Decomposition and Regeneration of Nitrogenous Organic Matter in Sea Water 285

PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1939 297